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LIVER FUNCTION MARKERS and OBESITY-ASSOCIATED PHENOTYPES: GENETIC AND ASSOCIATION STUDIES

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The primary goal was to study the influence of adipocyte number and volume, inflammation, insulin resistance, and genetic factors on indicators of liver injury, surrogate marker of non alcoholic fatty liver disease (NAFLD). The secondary goal was to explore the occurrence of NAFLD and its relationship with variations in liver function biomarkers. The first objective was to determine the association of plasma levels of monocyte chemoattractant protein-1 (MCP-1) with omental adipocyte number, insulin resistance and circulating concentrations of liver injury markers, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in unrelated baboons. Significant associations of MCP-1 with other measured traits were established. The second objective was to examine if adiposity-related parameters are under genetic influence and to evaluate their genetic correlations with AST in pedigreed baboons. Adipocyte volume and number, body weight and plasma AST were heritable. Genetic correlations between

adiposity-related phenotypes and AST were significant. A genome wide scan yielded a strong signal for adipocyte volume on chromosome 6. The third aim was to explore the genetic factors that influence variations in plasma levels of γ glutamyl transferase (GGT) and albumin (ALB), and to evaluate their genetic correlations with cardiovascular risk factors in pedigreed baboons. Significant linkages for GGT and albumin were identified on chromosome 20_22 and chromosome 10, respectively. Genetic correlations between ALB and cardiovascular risk factors were significant. No statistically significant associations were found between GGT and cardiovascular-related phenotypes. The fourth objective was to investigate the prevalence of NAFLD and its association with altered liver protein levels in unrelated baboons. The influence of weight and insulin resistance on the occurrence of NAFLD was inconclusive. Significant relationships between the variations in plasma levels of liver injury biomarkers and severity of the disease could not be established. In conclusion, the first three studies provided observational and genetic evidence of a relationship between liver function markers and adiposity-related factors in baboons. However, the results of the fourth study do not provide conclusive evidence to suggest that body weight and insulin resistance play a significant role in the development of NAFLD in these baboons.

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Chapter 1

Review of Literature

The National Health and Nutrition Examination Survey (1999-2002) estimates that more than 60 million adults (≥20years) are obese [Body mass index (BMI) ≥ 30 kg/m^2] (CDC, 2004). The prevalence of fatty liver disease in the adult population of the United States is 31 % (Browning et al., 2004); and is higher in the obese (Bellentani et al., 1994). Hepatic fat deposition in obese and insulin resistant individuals is called non-alcoholic fatty liver (NAFL) because alcohol use is not an etiologic factor (Festi et al., 2004). In non-alcoholic fatty liver, insulin resistance is believed to be the primary reason for the initiation of fat accretion in the liver cells (Charlton, 2004). Fatty liver is characterized histologically by macrovesicular steatosis, or accumulation of large lipid droplets in more than 5% of hepatocytes (Neuschwander-Tetri and Caldwell, 2003). This disease has clinical significance because it progresses to hepatocellular inflammation (steatohepatitis), which leads to liver cirrhosis (Cortez-Pinto & Camilo, 2004).

Hepatic steatosis is accompanied by altered circulating levels of liver proteins that are biomarkers of liver injury. However, genetic factors also are known to influence liver function markers (Bathum, 2001), as well as obesity-related factors (Comuzzie, 2002). It is hypothesized in the present project that common genes might affect both traits. In this study the contribution of indicators of liver injury, inflammation, insulin resistance, and genetic factors to non-alcoholic fatty liver disease will be explored.

1.1 Aims

1) To investigate the association of plasma levels of monocyte chemoattractant protein - 1 (MCP-1) with an indicator of insulin sensitivity and circulating levels of liver function markers in baboons.

   Hypothesis: Increased plasma levels of MCP-1 are associated with reduced insulin sensitivity and elevated plasma concentrations of liver enzymes.

2) To conduct univariate and bivariate analyses for omental adipocyte volume and number and plasma levels of aspartate aminotransferase in baboons.

   Hypothesis: There is a significant contribution of genes on omental adipocyte volume and number and plasma aspartate aminotransferase. A common genetic denominator may be responsible for relationship between these phenotypes.

3) To identify quantitative trait loci (QTLs) for circulating levels of γ glutamyl transferase (GGT) and albumin (ALB).

   Hypothesis: There is a substantial effect of specific chromosomal regions on plasma levels of GGT and ALB and on the genetic relationship between the traits related to liver function and cardiovascular disease.

4) To explore the occurrence of NAFLD and determine its relationship with markers of liver injury in baboons.

Hypothesis: The incidence of NAFLD will be higher in obese versus non-obese baboons. Also, markers of liver injury will be positively related to the extent of liver damage due to the disease.

1.2 Background

Hepatic steatosis was considered an aftermath of excessive alcohol consumption in the past, but now it is observed in obese individuals with minimal alcohol intake (Bradbury, 2006). Based on the 2000 census report (Census, 2001), 30 million obese adults in the United States are estimated to have steatosis (Angulo, 2002). Non-alcoholic fatty liver disease (NAFLD) is the most common liver pathology affecting the population of the Western countries (Falck-Ytter et al., 2001). This disease encompasses histological changes such as fatty liver, steatohepatitis, fibrosis and cirrhosis (Matteoni et al., 1999). The prevalence of hepatic steatosis and non-alcoholic steatohepatitis (NASH) in the general population is 20-30 % and 2-3%, respectively (Yu and Keeffe, 2002).The incidence of hepatic steatosis is far greater (50-70%) in obese individuals (Wanless and Lentz, 1990). Of these, about 20-30 % will develop NASH and 2-5 % will have cirrhosis (Reid, 2001). Furthermore, NAFLD has been implicated in the pathogenesis of hepatocellular carcinoma (Bugianesi et al., 2002).This proposal will focus on non-alcoholic fatty liver due to obesity and insulin resistance. Body mass index (Angulo et al., 1999) and insulin resistance (Marchesini et al., 1999) are independent predictors of the degree of triglyceride accumulation in the liver.

Although the mechanism leading to accumulation of liver triglycerides is unclear, it is postulated that the genesis of fatty liver is initiated by proinflammatory cytokines that

lead to insulin resistance (Choi & Diehl, 2005). It is believed that in obese individuals insulin resistance and inflammation are connected closely since obesity is characterized by a chronic state of inflammation. In the past adipocytes were thought to be a contributor to the pool of circulating immunomodulatory proteins. However, an investigation by Weisberg et al. (2003) has suggested that inflammation associated with obesity may be due to macrophage infiltration into adipose tissue. A transplantation study in transgenic mice showed that the macrophage population within the adipose tissue is derived from bone marrow (Weisberg et al., 2003). Curat et al. (2004) also have provided evidence of increased chemotaxis of monocytes into the adipose tissue, increasing the resident population of macrophages in the fat depots.

It is hypothesized (Arner, 2005) that changes in the fat cell size creates trauma within the fat depot. This trauma leads to the induction of MCP-1 (Juge-Aubry et al., 2005). It is postulated that MCP 1, a chemokine, is responsible for attracting monocytes into the visceral and subcutaneous fat depots of humans (Brunn et al., 2005). Within the monocytes nuclear factor kappa B (NF-κB), a proinflammatory transcription factor, is activated by tumor necrosis factor α (TNF-α) derived from adipocytes (Ghanim et al., 2004). Nuclear factor κB complex consists of two subunits, p65 (RelA) and p50, that are bound to a nuclear factor Kappa-B inhibitor (IκBα). This antagonist keeps the complex inactive in the cytoplasm (May and Ghosh, 1997) . Phosphorylation of IκBα by kinase IKKβ allows NFκB complex to translocate into the nucleus and turn on the transcription of inflammatory genes such as TNF-α and interleukin 6 (IL-6) (Bonizzi and Karin, 2004). However, NFκB is in turn stimulated by TNF-α, inducing a continuous cycle of inflammatory signals (Wellen and Hotamisligil, 2003). The pro-inflammatory cytokines

TNF-α and IL-6 from the monocytes that infiltrate the adipose tissue cause local insulin resistance by impeding the insulin-signaling pathway (Vettor et al., 2005). In summary, all of the above factors contribute to insulin resistance, the proposed metabolic basis for the occurrence of fatty liver in obese individuals.

Insulin resistance leads to the release of free fatty acids from the adipocytes into the circulation (Bugianesi et al., 2005). Normally, insulin inhibits the action of hormone sensitive lipase in the fat cells, thus, limiting the conversion of triglycerides to free fatty acids (Festi et al, 2004). When insulin action within the fat cells is impaired , the inhibitory effect of this protein on hormone sensitive lipase is removed; thereby, increasing the release of free fatty acids from fat stores into the blood (Arner, 2005). Both elevated fatty acids levels, and hyperinsulinemia (Yu and Ginsberg, 2005) characterize insulin resistance. One of the key features of steatosis is increased delivery of free fatty acids to the liver. Sources of these free fatty acids are lipolysis from subcutaneous and visceral adipocytes (Medina et al., 2004). However, the impact of non-esterified fatty acids from visceral adipocytes is the most significant. These acids from the central adipose depots drain directly into the liver via the portal vein (Kabir et al., 2005). In a study by Hsieh & Yoshinaga (1995) the prevalence of fatty liver was greater in normal weight individuals with a high waist to hip ratio, as compared to individuals with a low ratio [30.5% vs. 15.7%].

In hepatocytes high insulin levels increase free fatty acid synthesis, suppress free fatty acid oxidation and reduce triglyceride export (Sanyal, 2005). The mechanism by which insulin augments triglyceride accumulation in the liver is via up regulation of the transcription of Sterol Regulatory Element–Binding Protein -1c (SREBP). This protein

stimulates the expression of genes involved in synthesis of fatty acids (Richardson et al., 2005). Increased fatty acid concentration within the liver inhibits carnitine palmitoyl transferase-1 (CPT-1), the enzyme that transports fatty acids into the mitochondria for oxidation (Browning and Horton, 2004). In addition, insulin reduces triglyceride export from the liver in animal models by decreased secretion of VLDL, a triglyceride containing lipoprotein assembled in the liver (Au et al., 2004). This inhibitory effect is mediated by SREBP 1-c (Sato et al., 1999).

Many methods can be employed to diagnose NAFLD but liver biopsy is the only procedure that can truly distinguish the different stages of this disease (Hubscher S, 2004). The extent of histological changes associated with inflammation can be graded accurately with a biopsy (Adams and Talwalkar, 2006). Non-invasive methods used to detect the severity of liver damage due to fatty acid deposition include ultrasound (Joy et al., 2003), computer tomography (Limanond et al., 2004), magnetic resonance imaging (Saaseh et al., 2002) and liver injury markers (Schindhelm et al., 2006). Yet none of these techniques has the sensitivity and specificity as great as that of liver biopsy (Sanyal A, 2002). However, it is not feasible to perform liver biopsy for all patients suspected to have NAFLD due to cost, invasiveness, and risk associated with biopsy. Therefore, this study uses liver function marker as surrogate of NAFLD. Thus, the first aim of this research project is to investigate the association of plasma levels of monocyte chemoattractant protein -1 (MCP-1) with indicator of insulin sensitivity and circulating levels of liver function markers in baboons.

Monocyte chemotactic protein (MCP-1/CCL2) also is called the small inducible cytokine A2 (SCYA2), as well as monocyte chemotactic and activating factor (MCAF).

Endothelial cells, vascular smooth muscle cells and monocytes produce this low molecular weight (8 kDa) polypeptide on exposure to inflammatory stimuli (Becker, 2005). This peptide mediates its action by binding to its specific receptor, CC chemokine receptor 2 (CCR2) (Neels and Olefsky, 2006). Chemokines such as MCP-1 inhibit adipocyte differentiation and increase leptin production, acting as anti-adipogenic agents (Gerhardt et al., 2001).

Dahlman et al. (2005) showed that obese individuals have increased expression of chemokines in their adipocytes. The development of insulin resistance in mature fat cells may contribute to increased MCP-1 concentration (Sartipy & Loskutoff, 2003). It has been shown recently that reduction in MCP-1 mRNA levels improves insulin sensitivity in human adipose tissue (Di Gregorio et al., 2005). Patients with low insulin sensitivity were treated with thialzolidinedione, an antidiabetic drug, for ten weeks. The treatment ameliorated insulin resistance by decreasing MCP-1 mRNAs in the adipose tissue by 50%. A concurrent reduction in macrophage numbers also was observed. This proposal will investigate the genetic factors that control the expression of MCP-1. This knowledge will increase the understanding of insulin resistance related to obesity that results in hepatic steatosis.

Liver pathology leads to the release of their constituents into the circulation. The liver proteins whose plasma levels are abnormal in the event of hepatic injury are alanine aminotransferase, asparate amniotransferase, gamma-glutamyl transpeptidase and albumin. Other liver function aberrations such as elevated alkaline phosphate, bilirubin and lactate dehydrogenase are not evident in patients with a fatty liver (Charlton, 2004). The enzyme, alanine aminotransferase, catalyzes the reversible conversion of L-alanine

and alpha-ketoglutarate to L-glutamate and pyruvate. The transfer of an amino group from glutamate to oxaloaceate forms alpha-ketoglutarate and aspartate. This reaction is promoted by the second enzyme, asparate amniotransferase. The third marker is the catalyst gamma-glutamyl transpeptidase, which assists in the transfer of the glutamyl moiety cleaved from one protein or peptide to another. Finally, albumin, a negative acute-phase protein which constitutes about half of the serum protein, acts as a carrier for various intrinsic and extrinsic compounds including fatty acids. It also participates in stabilizing extracellular fluid volume.

Although other organs produce ALT and AST, liver houses the highest concentration of these aminotransferases. Low amounts of AST can be found in the heart, brain and red blood cells, whereas, skeletal muscle and kidney contains small concentrations of both the enzymes (Wroblewski, 1958). Similarly, GGT is present in miniscule quantities in biliary epithelial cells, renal tubules, intestine and the pancreas besides hepatocytes (Giannini et al., 2005). Liver is the major site for albumin production, and its reduced blood levels indicate impaired liver synthetic activity (Tessari, 2003).

In the Western world, NAFLD is the most common reason for altered levels of hepatic proteins according to a survey conducted by the NHANES III (Harrison et al., 2002). In a study by Chang et al., (2007) elevated ALT was estimated to be an independent predictor of NAFLD in healthy men. A significant correlation between ALT and hepatic fat accumulation, as determined by magnetic resonance spectroscopy, was established by Westerbacka et al. (2004) in both non-diabetic men and women. In humans, obesity and insulin resistance are known to be positively associated with raised

levels of plasma AST and GGT (Marchesini, 2005). In patients who lost weight after a laparoscopic gastric banding surgery the plasma levels of AST and GGT showed a marked reduction, which was parallel to improvement in histological features of fatty liver disease (Dixon et al., 2006). Angulo et al. (2007) found that serum albumin is one of the independent indicators of advanced stages of NAFLD. These results imply that hepatic proteins provide relevant information about hepatic pathology related to obesity and reduced insulin sensitivity.

In this research proposal it is hypothesized that genetic components may be responsible for variations in adipocyte volume and number as well as levels of liver enzymes. The second aim of this dissertation is to conduct univariate and bivariate analyses for omental adipocyte volume and number and plasma levels of aspartate aminotransferase in baboons. Adipose tissue expansion during weight gain can occur either by hypertrophy or hyperplasia, or both. In other words, enlargement of the fat depots could be due to increase in fat cell volume and/or size (Hausman et al., 2001). The balance between lipogeneis and lipolysis determines the adipocyte volume, and fat cell number is dictated by the equilibrium between the proliferation or differentiation and apoptosis (Avramm et al., 2005).

In adults initiation of obesity is believed to be distinctly because of fat cell hypertrophy. However, it is theorized that these cells have limited capacity to expand (Kawada et al., 2001). After reaching a critical mean volume the enlarged cell undergo death due to stress (Monteiro et al., 2006). After which fat depots grow due to hyperplasia (Coppack, 2005). The dead adipocytes activate inflammatory signaling pathways, subsequently, attracting macrophages into the adipose tissues to clear cellular

debris (Weisberg et al., 2003). The cytokines released by the macrophages compromise the insulin sensitivity of the fat cells (Cinti et al., 2005). Ultimately, inflammation-induced insulin resistance results in NAFLD.

It is postulated that NAFLD and cardiovascular disease might be associated since they share the same risk factors such as central obesity and compromised insulin sensitivity. Fatty liver disease is known to exacerbate insulin resistance as well as lead to dyslipidaemia, both of which contribute to atherosclerosis (Adiels et al., 2006). In addition, later stages of this hepatic condition are related to increased oxidative stress. The reactive oxygen species generated due to fatty acid oxidation can induce endothelial damage (Madamanchi et al., 2005). A recent observational study reported that NAFLD is a predictor of cardiovascular disease in healthy men and women. The relationship between NAFLD and prospective occurrence of CVD was significant even after inclusion of known cardiovascular risk factors in the multivariate model (Hamaguchi et al., 2007). Many epidemiological studies have shown strong associations between mildly elevated liver enzymes and increased CVD incidence (Wannamethee et al., 1995; Ruttmann et al., 2005; Ioannou et al., 2006; Schindhelm et al., 2007). These findings warrant an investigation into the influence of genetic factors on both these obesity related co-morbidities. Therefore, the third aim of this study is to identify quantitative trait loci (QTLs) for circulating levels of γ glutamyl transferase (GGT) and albumin (ALB).

The fourth aim of this project is to explore the occurrence of NAFLD and determine its relationship with markers of liver injury in baboons. Baboons will be the animal model used to explore the relationship between the phenotypes of interest. This primate is a well-established model for studying genetic of polygenic diseases (Cai et al., 2004;

Tejero et al., 2004) because baboon and human chromosomal structure are 96% homologous (Cole et al., 2003). Based on this information, a 10-cM linkage map for baboons using human microstatellite markers has been formulated (Rogers et al., 2000). A subset of the baboons in the population at Southwest Foundation for Biomedical Research (SFBR) in San Antonio, Texas have developed obesity (10%) and become insulin resistant (4%), in spite of the same controlled housing and diet. Most of these animals exhibit increased abdominal fat deposition, which is associated with insulin resistance and fatty liver (Comuzzie et al., 2003). Obesity related phenotypes such as weight (kg), fat mass (kg), fat free mass (kg) and leptin (ng/ml) are known to be highly heritable in these baboons and are almost identical to that reported in humans (Comuzzie & Allison, 1998). Thus, these baboons are ideal to study the effect of genetics on variability of liver function. Futhermore, baboons have been used in the study of alcohol-induced fatty liver (Lieber, 1975) and the effects of inflammation on hepatocytes (Lue et al., 1981).

In conclusion, the goal of this project is to establish the association between adipocyte volume and number, inflammation, insulin resistance and markers of liver damage. The secondary purpose is to identify the quantitative trait loci influencing the adiposity related traits and biomarkers of liver function In addition; this study also will explore the genetic relationship between surrogate markers of NAFLD, fat cell volume and number and cardiovascular risk factors.

Chapter 2

Association of Monocyte Chemoattractant Protein-1 with Adipocyte Number, Insulin Resistance and Liver Function Markers

2.1 Abstract

Monocyte chemoattractant protein-1 (MCP-1) is an inflammatory chemokine known to induce adipocyte dedifferentiation and insulin resistance. Both reduced insulin sensitivity and obesity have been implicated in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). The objective of this paper is to determine the association of plasma levels of MCP-1 with omental adipocyte number, insulin resistance and the circulating concentrations of liver injury markers, alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Fasting plasma from 43 baboons were assayed for MCP-1, insulin, glucose, ALT, and AST by standard procedures. Adipocyte number and volume were measured via biopsies of omental adipose tissue. The homeostatic model assessment method (HOMA) was used to estimate systemic insulin resistance. Mean weights of males and females were 32.42 ± 1.6 kg and 19.22 ± 0.8 kg. Sex and age adjusted correlations were significant for MCP-1 with adipocyte number ($r = -0.42$; $p = 0.01$), adipocyte volume ($r = 0.38$; $p= 0.02$), HOMA ($r = 0.45$; $p = 0.004$), ALT ($r = 0.46$; $p = 0.03$) and AST ($r = 0.45$; $p = 0.03$). These results suggest that MCP-1 is related with adipocyte dedifferentiation and systemic insulin resistance, ultimately resulting in NAFLD.

2.2 Introduction

In obesity adipocytes expand in size due to the deposition of triglycerides (Greenberg and Obin, 2006). However, this expansion may be limited. Chen et al. (2006) hypothesized that excessive hypertrophy of the fat cells might create a hypoxic environment within the adipose tissue. Consequently, this reduced availability of oxygen could lead to adipocyte death (Neels and Olefsky, 2006) which may then trigger macrophage infiltration to clear the cellular debris (Cinti et al., 2005). It is believed that monocyte chemoattractant protein-1 (MCP -1), a chemokine released by adipocytes, is responsible for attracting monocytes into these injured fat depots (Christiansen et al., 2005).

The expression and secretion of MCP-1 is known to be elevated during obesity in both mice (Takahashi et al. 2003) and humans (Bruun et al., 2005). MCP-1 mediates its action by binding to its specific receptor CC chemokine receptor 2 (CCR2) (Charo et al., 1994). In a recent study, obese mice lacking CCR2 had low macrophage concentrations in adipose tissue (Weisberg et al., 2006). In addition, MCP-1 is believed to induce the dedifferentiation of adipocytes, which might result in obesity- related disorders due to ectopic fat deposition (Tsuchiya et al., 2006). The expression of MCP-1 is higher in visceral, as compared to subcutaneous, adipose tissue (Bruun et al., 2005). This protein impairs insulin-stimulated glucose uptake in adipocytes, resulting in insulin resistance (Sartipy and Loskutoff, 2003). Thus, it is postulated that MCP-1 may play an integral role in the pathogenesis of non-alcoholic fatty liver disease (NAFLD) (Kanda et al., 2006).

Two major risk factors of NAFLD are visceral obesity and insulin resistance (Angulo P, 2002). In the pathogenesis of NAFLD in overweight and obese individuals,

insulin and excessive free fatty acids from insulin-resistant omental adipose tissue drain directly into the liver via the portal vein. In the liver, insulin up-regulates the conversion of unesterified fatty acids to triacylgycerols by the activation of sterol regulatory binding protein -1C (Kotani et al., 2004). Simultaneously, insulin reduces triglyceride export from the liver by decreased secretion of VLDL, a triglyceride-containing lipoprotein assembled in the liver (Au et al., 2004). This results in lipid accumulation in the hepatocytes that further triggers a cascade of necroinflammatory changes such as steatohepatitis, fibrosis and finally, cirrhosis (Day and James, 1998).
Ultimately, complications of NAFLD can lead to end stage liver disease and hepatocellular carcinoma (Neuschwander-Tetri BA, 2007).

At present, the gold standard for diagnosing fatty liver disease is a biopsy (Brunt, 2005). However, two biochemical markers of hepatic injury, plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) will be used in this study as non-invasive surrogates for NAFLD (Suzuki et al., 2006).

The purpose of this paper is to evaluate the importance of inflammation in the pathogenesis of NAFLD, using overweight and obese baboons as a model of chronic inflammation. We hypothesize that increased circulating levels of MCP-1 are associated with fewer mature adipocytes. This inflammatory molecule might also result in insulin resistance and elevated circulating levels of liver function markers due to subsequent liver injury caused by fat deposition.

2.3 Materials and methods

Animals

Forty three (31 females, 12 males) unrelated baboons (*Papio hamadrayas cynocephalus*) were included in this study The baboon colony is maintained at the Southwest National Primate Research Center located at the Southwest Foundation for Biomedical Research (SFBR) in San Antonio, TX, USA. These animals are gang-housed and fed a low fat, standard monkey chow diet *ad libitum* (Harlan Teklad 15% monkey diet, 8715, Indianapolis, IN).

Sampling and Analyses

Animals were fasted overnight (12 hours) and sedated with ketamine before collection of blood samples. Body weights were measured on a calibrated electronic scale (GSE, Chicago, IL). A total of 10 ml of blood was drawn from the antecubital vein in heparin tubes. Plasma was obtained by centrifugation at 2000 x g for 10 minutes and was stored in aliquots at –80 ^{0}C for future analysis. The Institutional Animal Care and Use Committee of the SFBR approved all procedures. Assays for ALT and AST were conducted by standard laboratory techniques using an Alfa Wasserman ACE clinical chemistry instrument (West Cladwell, NJ). Glucose was analyzed on an Analox spectrophotometer by the glucose oxidase method. Insulin and MCP-1 was measured by chemiluminescence in a Luminex 100 with endocrine multiplex immunoassay (Linco Research, Inc). Insulin resistance was calculated by the homeostatic model assessment (HOMA) method (Matthews et al., 1985). Biopsies of omental adipose tissue were taken and adipocyte number and volume per gram of tissue was analyzed by methods previously described by Lewis et al. (1986). All samples whose replicates varied >5% variations were reanalyzed.

Statistical Analysis

Descriptive statistics and other analyses were conducted using SPSS (SPSS Inc., Chicago, IL; V 10.0). Results are expressed as mean (standard error of mean). Student's t- test was used to determine the differences between sexes. All variables, other than sex and age, were log transformed to obtain a Gaussian distribution. Linear regression was performed, controlling for the effect of age and sex to estimate the association of plasma MCP-1 with omental adipocyte number and volume, HOMA scores, and plasma ALT and AST. Residual analysis, using Cook's distance and leverage coefficients, was conducted to analyze the effect of any point on the estimated regression (Sokal and Rohlf, 1994).

2.4 Results

Table 2.1 illustrates the traits analyzed in the baboons. The study population was comprised of more than twice as many females as males. The mean body weight of females was lower than that of males. Female body weights ranged from 14 kg to 32 kg and that of the males from 28 kg to 47 kg. Females were significantly older and had reduced concentrations of plasma MCP-1 as compared to males. Adipocyte number and volume, HOMA and plasma levels of ALT were not substantially different between the sexes. However, differences between the circulating concentrations of AST approached statistical significance.

After adjusting for age and sex, plasma MCP-1 was negatively associated with adipocyte number (Figure 2.1) and positively associated with adipocyte volume ($r = 0.38$; $p= 0.02$), HOMA (Figure 2.2), ALT (Figure 2.3) and AST (Figure 2.4).

2.5 Discussion

This is the first study to demonstrate that plasma levels of MCP-1 are negatively associated with omental adipocyte number and positively associated with adipocyte volume, HOMA and liver function markers in baboons.

In adipocyte dedifferentiation loss of mature adipocyte phenotype occurs through the suppression of two transcription factors, CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator- activated receptor γ (PPARγ) (Schaffler et al., 2006). These factors act in concert to regulate the expression of adipogenic genes required to maintain mature fat cells in a differentiated state (Kirkland et al., 2002). Sartipy and Loskutoff (2003) have suggested that MCP-1 may cause a reduction in the number of fat cells by reducing the mRNA expression of PPARγ. In addition, Nadler et al. (2000) have reported that adipose tissue from obese mice exhibit a decreased number of C/EBPα and PPARγ transcripts. The down regulation of these adipogenic genes might be due to obesity-related inflammation.

It is believed that fewer mature adipocytes lead to over expansion of existing fat cells, causing stress within the adipose tissue and stimulating the inflammatory system (Heilbronn et al., 2004). Our data suggests that obesity-related inflammation may lead to an abnormal adipocyte life cycle, which reduces the number of lipid-bearing mature adipocytes and finally, lead to other metabolic diseases.

A positive relationship between plasma levels of MCP-1 and the insulin resistance index (HOMA) was observed in this study. This association has also been shown in mice and humans. For example, Kamei et al. (2006) demonstrated that over expression of MCP-1 in transgenic mice resulted in a systemic insulin resistance. In a study by Weisberg et al. (2006), insulin sensitivity improved in obese mice treated with an

antagonist of CCR2, the MCP-1 receptor. In overweight and obese humans who are prone to having low insulin sensitivity, the circulating levels of MCP-1 were positively related to HOMA scores (Kim et al., 2006). Thus, it is likely that MCP-1 may participate in the pathogenesis of insulin resistance by impairing insulin signaling and reducing insulin-mediated glucose uptake (Kamei et al., 2006).

We also found that circulating concentrations of MCP-1 were positively related to liver function markers, plasma ALT and AST. The levels of these liver enzymes are known to be elevated in obese and insulin-resistant individuals (Colicchio et al, 2005). MCP-1 may be associated with elevated levels of ALT and AST through its contribution to both insulin resistance and hepatic inflammation. Thus, the disruption of insulin signaling in adipocytes due to inflammatory molecules may lead to ectopic fat deposition (Raz et al., 2005). This triglyceride accumulation in the liver may then increase the susceptibility to hepatic inflammation and apoptosis. Subsequently, these events may lead to a release of hepatocyte constituents into the circulation and raising the blood levels of liver injury markers.

The serum levels of MCP-1 are elevated in individuals with steatosis, with the highest concentrations in subjects with non-alcoholic steatohepatitis (Haukeland et al., 2006). The association between MCP- 1 and NAFLD is strengthened further by the findings of Kanda et al. (2006) who showed that insulin resistance and hepatic steatosis were ameliorated due to the deletion of the MCP-1 gene in mice that were fed a high fat diet.

Although baboons have been used extensively for the study of alcoholic fatty liver disease (Navder et al., 1999; Savolainen et al., 1986; Lieber et al., 1985), this is the first

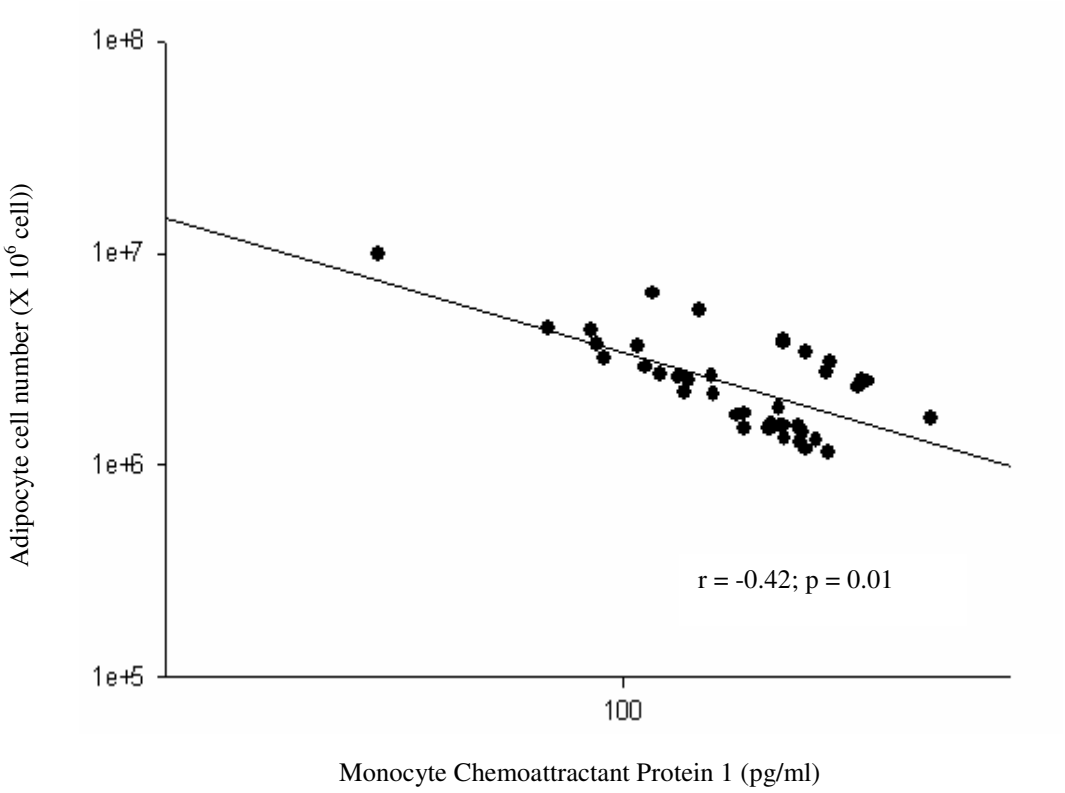
time that this primate has been used as a model for obesity-related, hepatic pathology. In summary, this paper suggests that elevated plasma levels of MCP-1 and reduced adipocyte cell number are inversely related. This endogenous, immunomodulatory chemokine might promote adipocyte dedifferentiation (reduced cell number) events that may interfere with insulin-mediated glucose uptake and subsequent insulin resistance. Ultimately, obesity-related inflammation, in conjunction with insulin resistance, may lead to the pathogenesis associated with NAFLD.

Table 2.1 Descriptive statistics of baboons *

Trait	Male	Female	p value
Number	12	31	
Weight (kg)	32.42 ± 1.6	19.22 ± 0.8	0.0001
Age (yrs)	14.35 ± 1.06	20.67 ± 1.0	0.001
Monocyte chemoattractant protein 1 (pg/ml)	229.68 ± 19.1	152.89 ± 8.9	0.0001
Adipocyte number ($X10^6$ cell)	$5 X 10^6 \pm 1 X 10^6$	$4 X 10^6 \pm 9 X 10^5$	0.60
Adipocyte volume (nm)	0.33 ± 0.1	0.55 ± 0.1	0.09
HOMA[a]	1.37 ± 0.4	1.59 ± 0.2	0.62
Alanine aminotransferase (IU/L)	32.41 ± 3.7	28.40 ± 3.1	0.47
Aspartate aminotransferase (IU/L)	29.25 ± 2.9	23.51 ± 1.5	0.06

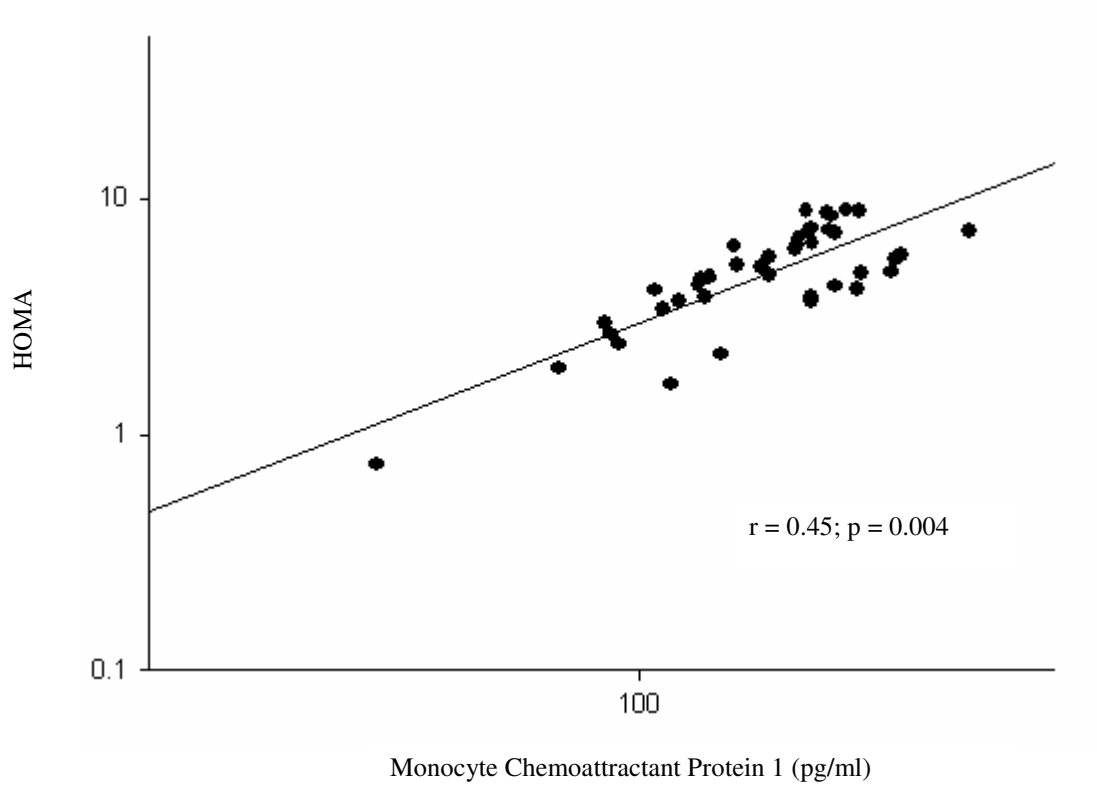
[a]Homeostasis model assessment method
* Mean (s.e.m)

Figure 2.1 The association between the plasma monocyte chemoattractant protein 1 and adipocyte cell number in baboons



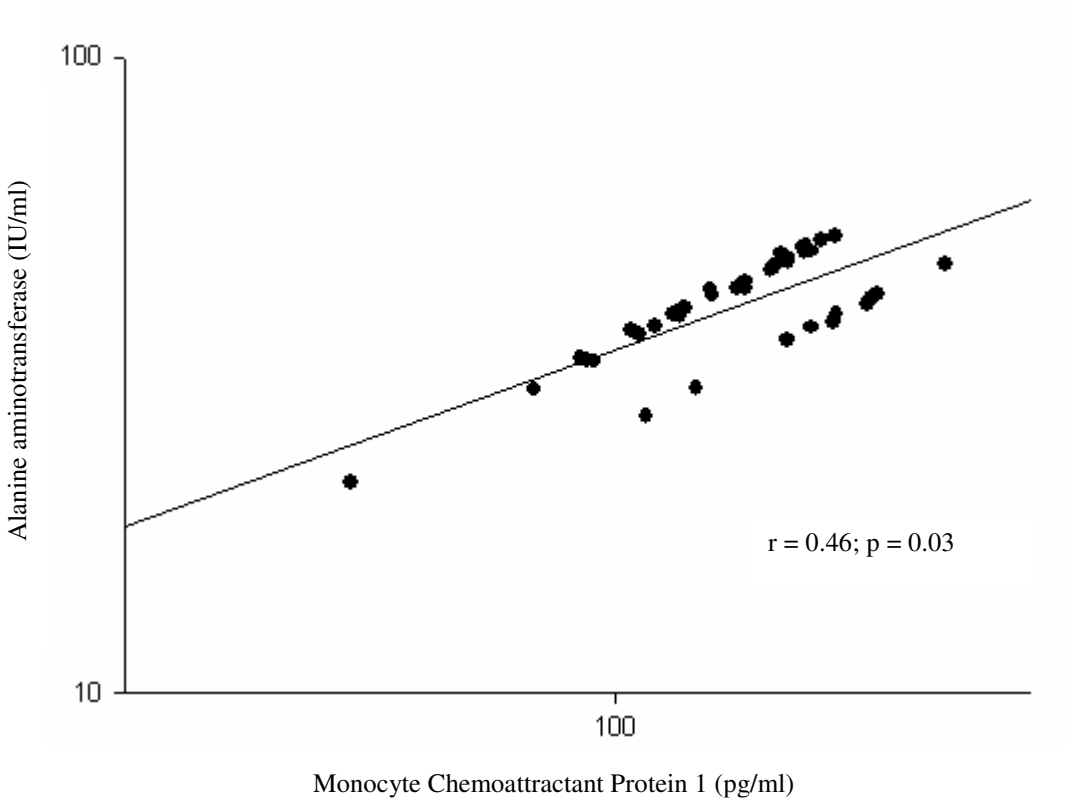
* Age and sex adjusted

Figure 2.2 The association between the plasma monocyte chemoattractant protein 1 and HOMA insulin resistance index in baboons



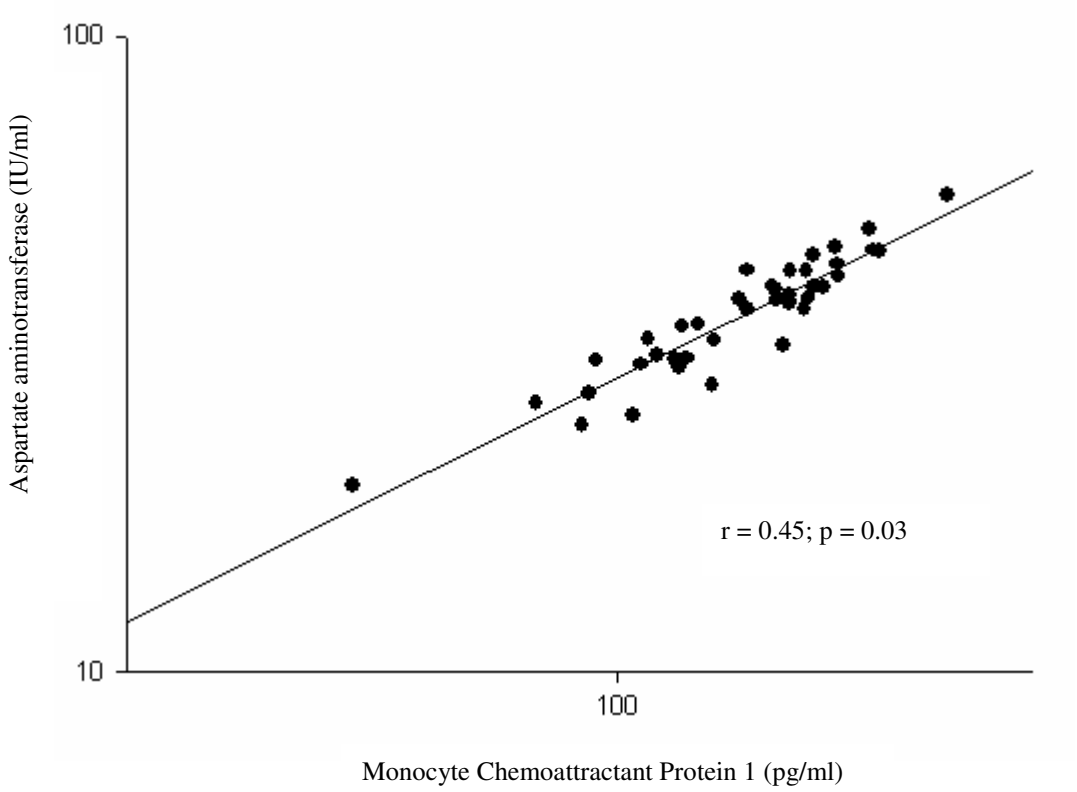
* Age and sex adjusted

Figure 2.3 The association between the plasma monocyte chemoattractant protein 1 and alanine aminotransferase in baboons



* Age and sex adjusted

Figure 2.4 The association between the plasma monocyte chemoattractant protein 1 and aspartate aminotransferase in baboons



* Age and sex adjusted

Chapter 3

Identification of a QTL for Adipocyte Volume and of Shared Genetic Effects between Adipocyte Volume and Number with Aspartate Aminotransferase

3.1 Abstract

Non-alcoholic fatty liver disease is a hepatic condition that affects a large number of overweight and obese individuals. Aspartate aminotransferase (AST) is one of the liver enzymes whose concentration is elevated in patients with visceral obesity. The purpose of this study was to examine if adiposity related parameters are under genetic influence and to evaluate their genetic correlations with AST.

Fasting plasma of 374 pedigreed baboons from the Southwest National Primate Research Center at the Southwest Foundation for Biomedical Research, San Antonio, TX were assayed for AST. Adipocyte volume and number were measured using biopsies of omental adipose tissue. Genetic analyses were conducted by a maximum likelihood based variance decomposition approach implemented in the computer program SOLAR. Adipocyte volume and number, body weight and plasma AST were highly heritable. Genetic correlations between the measured adiposity - related phenotypes and AST were significant. A genome wide scan yielded a strong signal for adipocyte volume on the baboon homologue of human chromosome 6 with a maximum LOD score of 3.2 at 73 cM near marker *D6S1028.* These results suggest that the size of omental adipocytes is under significant genetic control and that shared genetic factors influence adiposity associated traits and AST.

3.2 Introduction

In obesity, white adipose tissue enlarges due to increased adipocyte size (hypertrophy) and/or number (hyperplasia) (Hausman et al., 2001). Adipocyte size is a function of the balance between lipogenesis and lipolysis (Schling and Loffler, 2002). However, fat cell number is controlled by the equilibrium between proliferation (or differentiation) and apoptosis (Avramm et al., 2005). It is postulated that failure of preadipocytes to differentiate into mature lipid storage cells expands the existing adipocytes during periods of surplus energy intake (Heilbronn et al. 2004). The resultant hypertrophic cells are known to have dysfunctional lipid and glucose metabolism (Smith et al., 2006), leading to insulin resistance (Mc Garry and Dobbins, 1999) and ectopic fat accumulation in tissues other than adipose depots (Danforth E, 2000).

Most obesity related diseases are associated with hypertrophic adipocytes (Greenberg and Obin, 2006). The large fat cells are highly susceptible to apoptosis because of stress and consequently they attract mononuclear cells (Chen et al., 2005). The recruited macrophages secrete cytokines that impair insulin sensitivity of the surrounding fat cells within the tissue (Shi et al, 2006). The insulin resistance augments lipolysis, resulting in elevated concentrations of unesterified fatty acids in the circulation. Consequently, high plasma levels of insulin and free fatty acids may induce hepatic steatosis by the up regulation of the synthesis and accumulation of triglycerides in the liver (Mendez-Sanchez et al., 2006).

It is hypothesized that omental adiposity is directly related to non-alcoholic fatty liver disease (NAFLD) (Adams and Angulo, 2005) because blood supply from visceral adipose tissue drains directly into the liver via the portal vein. Donelley et al. (2005) have

shown that 60% of the triglycerides accumulated in the liver are derived from the visceral adipocytes. One of the features of NAFLD is elevated levels of liver enzymes. Aspartate aminotransferase (AST), a liver enzyme is the key focus of this paper as it is elevated in patients with visceral obesity and fatty liver disease (Cancello et al 2006). The variation in the circulating levels of this enzyme is under genetic influence in humans (Bathum et al., 2001). Also, it is well established that genetic factors contribute to obesity related phenotypes. Therefore, the goal of this study is to identify specific chromosomal regions that might affect adipocyte size and number and to determine if common genetic factors regulate these adiposity traits and AST in baboons.

Baboons from the colony at the Southwest National Primate Research Center were used as the animal model. Approximately 10% of these animals become obese spontaneously, in spite of similar diet and environmental conditions (Comuzzie et al., 2003). This finding implies that a strong genetic component influences the obesity related phenotypes in these animals. In terms of evolution, humans and baboons have similar DNA and gene sequences (Rogers and Hixson, 1997). For example, the similarity in their protein structure makes it possible to use human assay kits for the analysis of baboon proteins. Furthermore, both humans and baboons exhibit central obesity, with fat deposition occurring in the visceral adipose tissue (Comuzzie et al., 2003) and these animals exhibit the whole spectrum of histological changes associated with NAFLD (Bose et al, 2006). Moreover, it is more feasible to obtain omental adipose tissue biopsies from a large number of pedigreed baboons than it is in humans. Thus, baboons serve as an advantageous model for studying the genetic factors associated with obesity-related diseases.

3.3 Materials and methods

Animals

This study included 374 (254 females, 119 males) pedigreed baboons (*Papio hamadrayas)* housed at the Southwest National Primate Research Center located at the Southwest Foundation for Biomedical Research (SFBR) in San Antonio, TX, USA. These animals are gang-housed and fed a low fat standard monkey chow diet *ad libitum* (Harlan Teklad 15% monkey diet, 8715, Indianapolis, IN).

Sampling and Phenotypic analyses

Animals were fasted overnight (12 hours) and sedated with ketamine prior to collection of blood samples. Body weight was measured on a calibrated electronic scale (GSE, Chicago, IL). A total of 10 ml of blood was drawn from the antecubital vein in heparin tubes for analysis of AST. Plasma was obtained by centrifugation at 2000 x g for 10 minutes and was stored in aliquots at -80^0 C for future analysis. The Institutional Animal Care and Use Committee of the SFBR approved all procedures. Assay of AST was conducted by standard laboratory techniques using Alfa Wasserman ACE clinical chemistry instrument (West Cladwell, NJ). Omental adipose tissue biopsies were collected as previously described by Cole *et al.* (2003). Adipocyte volume and fat cell number per gram of tissue were analyzed by the method of Lewis (1986). All samples whose replicates had >5% variations were reanalyzed.

Genotyping

Using human PCR primers, homologous loci from baboon genomic DNA were amplified to construct a baboon genetic linkage map (Rogers et al., 2000). Electrophoresis was used to obtain baboon genotypes via ABI 373 and ABI 377 automated sequencers using fluorescently labeled primers. Genescan and Genotyper software (Applied Biosystems, Foster

City, CA) were utilized for genotype scoring. A map with an average marker density of 10 cM was created using 331 markers.

Statistical genetic methods

The maximum likelihood based variance decomposition method was used to perform the quantitative genetic analyses. The phenotypic variance of a quantitative trait was decomposed into additive genetic and nongenetic (environmental) components. The proportion of variance in a phenotype due to additive genetic effect was defined as heritability. (h^2). The likelihood ratio test compares the likelihood of a model whose h^2 was estimated to the likelihood of a model whose h^2 was restricted to zero. Twice the difference of the log likelihoods was asymptomatically distributed as ½: ½ mixture of chi-sqaure variable, with one degree of freedom and a point mass at zero (Self and Liang, 1987).

An extension of the univaraite model was used for bivariate genetic analyses. The bivariate phenotype is a result of the animal's phenotypic values, population means, the additive genetic estimates and environmental effects. This model was used to calculate the genetic and environmental variance-covariance matrices, in addition to genetic and environmental correlations. Both univariate and bivariate genetic analyses were conducted using the Sequential Oligogenic Linkage Analysis Routinues (SOLAR) computer program (Almasy and Blangero, 1998). Age, sex, age squared and their interactions were included as covariates for the analyses. Software program PEDSYS computed the group means of male and female baboons.

3.4 Results

The number of relative pairs studied in the sample analyzed is shown in Table 3.1. The descriptive statistics of the baboons according to sex are displayed in Table 3.2. There were twice as many females as males, and the female baboons were significantly older and had lower body weights. However, males had a higher concentration of plasma AST and smaller adipocyte volume. Adipocyte number did not vary significantly.

The heritabilities for body weight, plasma AST, and adipocyte volume and number are given in Table 3.3. All of these heritabilities were significant with body weight having the highest heritability of all the traits studied. Table 3.4 shows the genetic correlations between plasma concentration of AST and adiposity markers. Circulating concentrations of AST had a positive association with body weight and adipocyte volume and a negative relationship with adipocyte number.

The genome wide scan for adipocyte volume is shown in Figure 3.1. The strongest signal obtained was detected on the baboon homologue of human chromosome 6, with a maximum LOD score of 3.2 at 73 cM near marker *D6S1028* (Figure 3.2).

3.5 Discussion

This is the first study to identify a significant QTL for adipocyte volume and also report significant genetic correlations between AST and omental adipocyte number and volume and body weight. Aspartate aminotransferase (AST) also called glutamate oxaloacetate transaminase, is a pyridoxal phosphate-dependent enzyme that participates in amino acid metabolism as well as the urea and tricarboxylic acid cycles in the liver (Panteghini, 1990). The levels of this enzyme are elevated in obese subjects (Marchesini et al.,2005), presumably due to the NAFLD, which is highly prevalent in these individuals (Gholam et al., 2007). In these patients, visceral adiposity, coupled with the

presence of insulin resistance, may link obesity to fatty liver disease (Angelico et al., 2005).

Adipocytes comprise about 0.5 to 1 % of the total body cells. The weight of the adipocytes accounts for approximately 2-3 % of the body weight in a healthy person, as opposed to 30-40% in the obese (Prins and O'Rahilly, 1997). In obesity, hyperplasia and hypertrophy cause expansion of the adipose tissue mass. It is believed that during weight gain adipocytes increase in size to accommodate the newly synthesized triglycerides (Spiegelman and Flier, 1996). However, once a threshold size is achieved, the fat stores then expand due to differentiation of pre-adipocytes (Bjorntorp, 1996).

It is hypothesized that fat cells have a limited capacity to expand (Kawada et al., 2001). Once the enlarged cells reach a critical mean volume they are liable to rupture due to stress (Monteiro et al., 2006), after which fat depots expand due to hyperplasia. (Coppack, 2005). However, if the preadipocytes fail to proliferate and differentiate into mature fat cells it is plausible that this inability might lead to obesity related diseases (Bakker et al., 2006).

The dead adipocytes activate inflammatory signaling pathways. These pathways could compromise the insulin sensitivity of the remaining fat cells (Cinti et al., 2005). In addition, dead cells attract macrophages into the adipose tissue to clear cellular debris (Weisberg et al., 2003). These immune cells release cytokines into the milieu, which further exacerbates insulin resistance within the adipose tissue (Xu et al., 2003). The impairment of insulin signaling, in turn, stimulates lipolysis and increases the circulating concentrations of unesterified fatty acids (Permana et al., 2006). These free fatty acids that are released, particularly from the visceral fat depots, are transported to the nearby

organs such as the liver and may cause organ damage by initiating hepatic triglyceride accumulation (Eguchi et al., 2006).

In this study we found a significant genetic relationship between plasma levels of AST, body weight and adipocyte volume. These results imply that the same genetic factors may influence these adiposity-related traits and levels of AST. The negative association between circulating AST and adipocyte number suggest that a reduced number of mature adipocytes might lead to fat accumulation that damages the liver, thereby raising the blood levels of AST.

The identification of a QTL for omental adipocyte volume is the other significant finding of this study. Larger cells have been implicated in the development of type II diabetes (Weyer et al., 2000) and high plasma levels of non-esterified fatty acids (Paolisso et al., 1995). It has been shown that bigger fat cells have higher mRNA concentrations of enzymes involved in lipid synthesis and hydrolysis than do those of a smaller size (Farnier et al, 2002). Moreover, the hypertrophic adipocytes apear to be metabolically dysregulated.

Adipocyte size also affects the secretion of cytokines. Hypertrophy induces the release of pro-inflammatory adipokines which might be responsible for the chronic state of inflammation in obesity (Skurk et al., 2006). Fat cells with a larger volume produce significantly more chemoattractants which attract monocytes into the adipose tissue. The cytokines released by these immune cells negatively affect the differentiation of preadipocytes (Stephen et al., 1993) and promote apoptosis of the existing cells (Lin et al., 2004). Therefore, in addition to total fat mass, both the size and number of cells that

constitute the adipose tissue play a major role in the pathology of obesity related co-morbidites.

Two potential positional candidate genes present within one LOD support interval of the signal for adipocyte volume on Chromosome 6 are fatty acid binding protein (*FABP7*) (OMIM 602965) (Shimizu et al., 1997) and forkhead transcription factor (*FOXO3A*) (OMIM 602681) (Anderson et al., 1998). Fatty acid-binding proteins are small proteins that increase the transfer of fatty acids into the cell and enhance the catalytic action of enzymes involved in fatty acid metabolism. It is believed that FABP helps to maintain the systemic energy homeostasis by providing a critical link between lipid metabolism and cellular functions in adipose tissue and other organs (Maeda et al., 2005). The other candidate gene, forkhead transcription factor is stimulated due to stress and induces the expression of genes related to cell death. Its expression has been upregulated in rats fed a high fat diet suggesting that apoptotic pathways might be activated in obesity (Relling et al., 2006).

In conclusion, this study establishes that the volume and number of adipocytes are heritable. The bivariate genetic analyses demonstrate that AST levels, fat cell volume and number and body weight are regulated by common genetic factors. Future work should identify polymorphisms in the chromosomal region of the QTL for adipocyte cell number that might influence obesity related co-morbidities.

Table 3.1 Relative pairs in the analyzed sample

Relationship	Number
Unrelated	4187
Self	411
Parent-offspring	122
Siblings	260
Grandparent-grandchild	2
Avuncular	60
Half-siblings	2851
Half avuncular	616
1st cousins	1
Half 1st cousins	15
Half siblings & 1st cousins	2
Half siblings & half 1st cousins	62
Half siblings & half avuncular	8

Table 3.2 Descriptive statistics of baboons based on sex*

Phenotype	Male	Female	p value
Number	119	255	
Age (yrs)	12.17 ± 0.3	15.84 ± 0.3	0.0001
Body weight (kg)	31.50 ± 0.4	19.49 ± 0.2	0.0001
Aspartate aminotransferase (IU/L)	31.33 ± 0.8	26.55 ± 0.5	0.0001
Adipocyte			
Volume (nm)	0.39 ± 0.04	0.58 ± 0.02	0.0001
Number (x 10^6 cell)	4 X 10^6 ± 4 X 10^5	3 X 10^6 ± 4 X 10^5	NS

*Mean (s.e.m)

Table 3.3 Heritabilities of body weight, aspartate aminotransferase and adipocyte volume and number

Phenotype	Heritability ± s.e.m	p value
Body weight	0.70 ± 0.10	< 0.0001
Aspartate aminotransferase	0.37 ± 0.11	< 0.0001
Adipocyte		
Volume	0.30 ± 0.11	< 0.0001
Number	0.20 ± 0.11	< 0.05

Table 3.4 Genetic (rho_G) correlations between plasma concentration of aspartate aminotransferase and body weight, adipocyte volume and number

Phenotype	ρ Genetic	p value
Body weight	0.40	0.04
Adipocyte		
Volume	0.80	0.03
Number	-0.88	0.02

Figure 3.1 Genome-wide scan of adipocyte volume.
The y –axis denotes the chromosomal location and the x - axis represents the LOD score

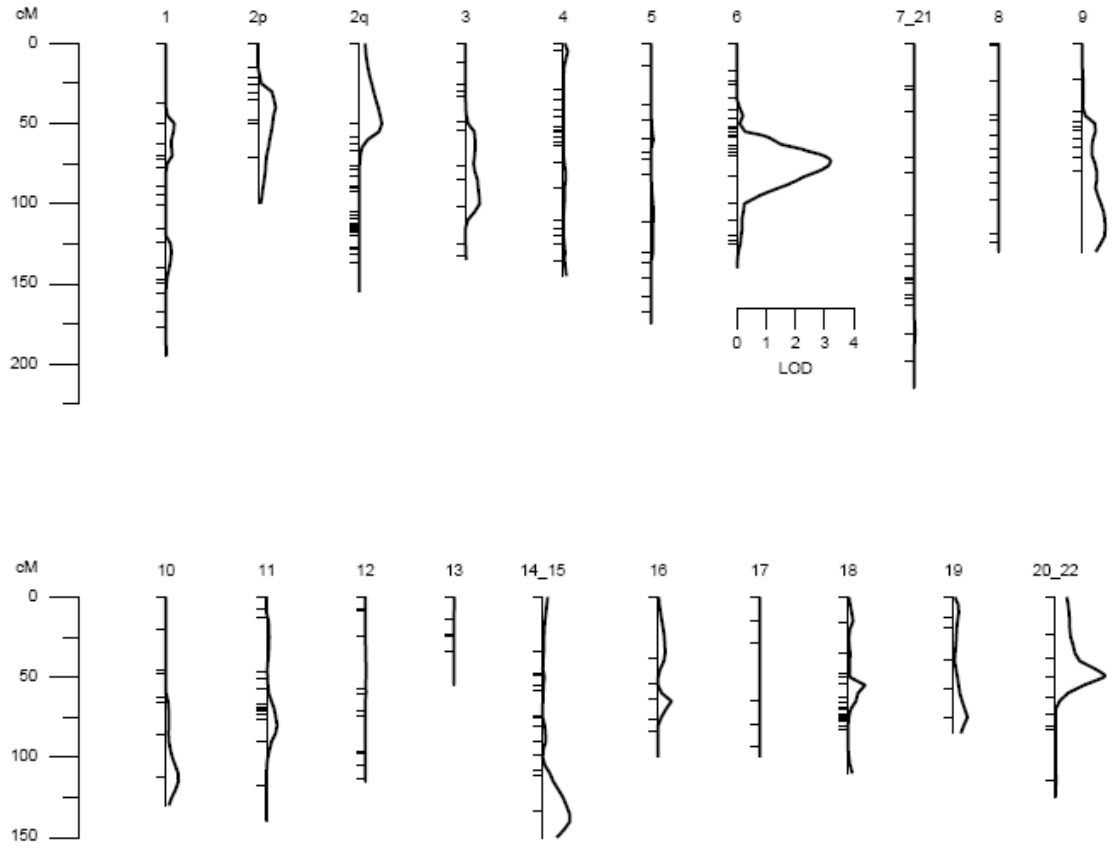
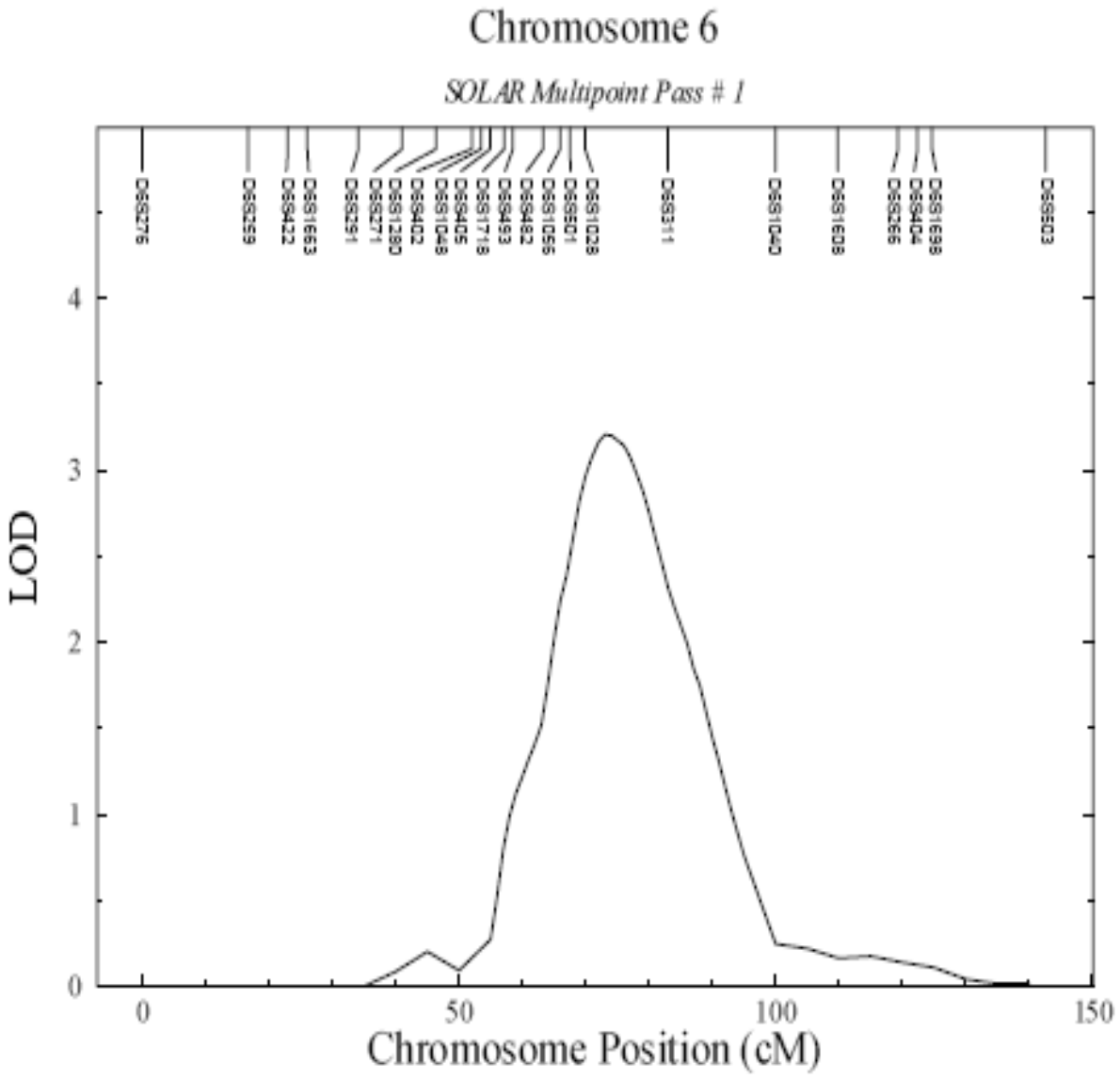


Figure 3.2 LOD plot for adipocyte volume on chromosome 6



Chapter 4

QTLs Regulating Plasma Levels of GGT and ALB and their Genetic Correlations with Cardiovascular Risk Factors

4.1 Abstract

Gamma glutamyl transferase (GGT) and albumin (ALB) are two markers of liver function. These two proteins have been associated with non-alcoholic fatty liver disease and cardiovascular disease. The objective of this study was to explore the genetic factors that influence variation in the plasma levels of GGT and ALB and to evaluate their genetic correlations with cardiovascular risk factors. Baboons from the Southwest National Primate Research Center at the Southwest Foundation for Biomedical Research, San Antonio, TX were used as an animal model. The baboons were fed a standard monkey chow diet *ad libitum*. Fasting plasma concentrations of GGT, ALB, triglycerides, total cholesterol and LDL cholesterol were measured in 350 pedigreed adult baboons by standard assay procedures. A maximum likelihood based variance decomposition approach implemented in the computer program SOLAR was used to conduct genetic analyses. The heritabilities of GGT (h^2=0.55; p< 0.0001) and ALB (h^2=0.42; p< 0.01) were significant. No statistically significant associations were found between GGT and the cardiovascular related phenotypes. Genetic correlations between ALB and cardiovascular risk factors were significant. A QTL (LOD = 2.8) for GGT plasma levels was identified on the baboon homologue of human chromosome 22 between markers *D22S304* and *D22S280*. A QTL (LOD =2.3) near marker *D10S1432* was detected on the baboon homologue of human chromosome 10 for ALB. These results imply that

variations in the plasma levels of GGT and ALB are under significant genetic regulation and that a common genetic component influences ALB and cardiovascular risk factor phenotypes.

4.2 Introduction

The leading cause of an abnormal liver function test in overweight and obese individuals is non-alcoholic fatty liver disease (NAFLD) (Ekstedt et al, 2006). This hepatic pathology encompasses simple steatosis, steatohepatitis, fibrosis and cirrhosis (Liou and Kowdley , 2006). In the pathogenesis of this disease, a "two-hit model" implicates obesity related factors such as visceral obesity, insulin resistance and dyslipidemia (Day, 2002). According to this model, the first hit takes place when monocytes infiltrate the omental adipose tissue to scavenge adipocytes that have undergone necrosis due to stress within the tissue (Cinti et al., 2005). The immune cells secrete cytokines in the milieu which disrupts the insulin signaling pathways of the surrounding adipocytes (Lumeng et al., 2006). Absence of the lipogenic control of insulin enhances lipolysis within the adipocytes. Consequently, elevated concentrations of free fatty acids and insulin are directed to the liver from the adipose tissue (Utzschneider and Kahn, 2006). The unesterified fatty acids accumulate in the liver as triglycerides because insulin upregulates the hepatic synthesis of triglycerides through activation of sterol regulatory binding protein (SREBP-1C) (Kotani et al., 2004). This ultimately leads to hepatic steatosis (Gaemers and Groen , 2006).

In the second hit steatosis leads to the advanced stages of NAFLD. Fat stored in the liver undergoes oxidation in the mitochondria which generates reactive oxygen species (Begriche et al., 2006). These free radicals result in lipid peroxidation, cytokine

induction and apoptosis, which lead to inflammation, fibrosis and cell death (Videla et al., 2006).

Epidemiological studies have shown a strong positive relationship between markers of NAFLD such as liver enzymes and prevalence of cardiovascular disease (CVD) (Schindhelm et al., 2006; Targher et al, 2006). The focus of this paper is on two indicators of liver function, gamma-glutamyl transferase (GGT) and albumin (ALB), both of which have also been implicated in the pathogenesis of atherosclerosis. It is known that the variation in the plasma level of these liver related proteins is genetically influenced. To explore these genetic factors and also to evaluate the relationship of these liver markers with cardiovascular risk phenotypes we have chosen the baboon as an animal model.

The pedigreed baboons from the colony maintained by the Southwest National Primate Research Center are an excellent model for this study since the availability of large extended pedigrees gives statistical power. Although all the baboons share similar diet and housing conditions, 10% become obese spontaneously and 4% become hyperglycemic (Comuzzie et al., 2001). Gene and protein sequence identity is conserved between baboons and humans and both are physiologically and developmentally similar. For example, during weight gain fat is deposited primarily in the abdominal area of baboons. It has been known that visceral adiposity is strongly linked to several metabolic diseases such as cardiovascular ailments, type 2 diabetes and fatty liver (Licata et al., 2006). Obese and insulin resistant baboons from this colony have been diagnosed with hepatic steatosis, steatohepatitis and fibrosis (Bose et al., 2006). In addition, baboons have been used for genetic studies related to atherosclerosis (Mahaney et al., 1999),

insulin-resistance (Cai et al., 2004), obesity (Tejero et al., 2005) and liver gene expression (Cox et al., 2006).

The purpose of this study is to identify chromosomal regions that affect the variation in the plasma levels of GGT and albumin and to determine the genetic correlations between these liver related proteins and cardiovascular related parameters.

4.3 Materials and methods

Animals

The study population consisted of 350 (254 females, 96 males) pedigreed baboons (*Papio hamadrayas*) from the colony maintained at the Southwest National Primate Research Center located at the Southwest Foundation for Biomedical Research (SFBR) in San Antonio, TX, USA. The baboons are gang housed and fed a standard monkey chow diet *ad libitum* (Harlan Teklad 15% monkey diet, 8715, Indianapolis, IN).

Sampling and Analyses

Blood samples were drawn after an overnight fast (12 hours) from animals sedated with ketamine. Heparin tubes were used to collect samples for analysis of GGT, albumin, triglycerides, total cholesterol and LDL cholesterol. The samples were subjected to centrifugation at 2000 x g for 10 minutes and the resultant plasma was stored in aliquots at –80C for future analysis. The animals were weighed on a calibrated electronic scale (GSE, Chicago, IL). The Institutional Animal Care and Use Committee of the SFBR approved all procedures.

Triglycerides, total cholesterol, LDL cholesterol, GGT and albumin were analyzed by standard laboratory techniques using the Alfa Wasserman ACE clinical chemistry instrument (West Cladwell, NJ). Samples whose replicates deviated >5% were reanalyzed.

Genotyping

A baboon genetic linkage map was constructed by amplifying homologous loci from baboon genomic DNA using human PCR primers (Rogers et al., 2000). Electrophoresis of PCR products occurred on ABI 373 and ABI 377 automated sequencers using fluorescently-labeled primers, and genotypes were scored using Genescan and Genotyper software (Applied Biosystems, Foster City, CA). A map with an average marker density of 10 cM was generated using 331 markers.

Statistical genetic methods

The quantitative genetic linkage analyses for the target phenotypes were conducted using the variance decomposition methods implemented in the computer package SOLAR (Almasy and Blangero, 1998). The variance component approach incorporates all of the genetic linkage information in the extended pedigrees, assuming all possible biological relationships simultaneously. Power-diminishing, simplifying assumptions that are integrated in most "penetrance model free" methods are relaxed in the extensions of this method. The variance component method is more efficient since it requires the estimation of fewer parameters than the fully parametric penetrance-based linkage methods.

The variance component linkage method is based on classical quantitative genetic principles, in which the phenotype is assumed to be influenced by both genetic and environmental factors. Unlike the classical models, the genetic factors can include specific loci at defined chromosomal locations. Evidence for genes is obtained from the observed covariances (or correlations) among different classes of relatives. The *a priori* structure for these correlations among individuals in a family is provided by a matrix that contains the

coefficients of relationship for all pairs of individuals, which also are the prior probabilities that two individuals will share a particular allele identical-by-descent.

In the variance component approach, the observed covariances among individuals are compared to expected covariances obtained under the linkage model including both the locus-specific and genomic factors, each of which is quantified by a variance component. If the variance component for a specific chromosomal location is significantly greater than zero, there is evidence for a locus influencing the phenotype at that location. Evidence for such a locus requires that the statistical "fit" of the observed phenotypic covariances and the expected covariances (given the linkage model under consideration) be significantly better when the locus-specific component is allowed than when it is not.

Parameter estimation and hypothesis testing are performed using a likelihood framework, assuming multivariate normality as a working model within pedigrees. For each phenotype, we will test the null hypothesis that the additive genetic variance due to a specific QTL equals zero (no linkage) by comparing the likelihood of this restricted model with that of a model in which the variance due to this QTL is estimated.

The difference between the two $\log_{10}$ likelihoods yields a LOD score, which measures the support for the hypothesis of linkage over that of "no linkage" at a given chromosomal location. A LOD score of 3.0, for example, indicates that the statistical support for the linkage hypothesis is 10^3 times more than for the null hypothesis. P-values for this test are obtained from twice the difference in $\log_e$ likelihoods of these two models, which yields a test statistic that is asymptotically distributed as a (1/2):(1/2) mixture of a chi square variable and a point mass at zero (Self and Liang, 1987).

4.4 Results

Information describing the number of relative pairs represented in the sample for these analyses is provided in Table 4.1. Descriptive statistics of the phenotypes according to sex are given in Table 4.2. Male baboons were younger and had higher body weights than females due to the sexual dimorphism in these animals (Tejero et al., 2004). Female baboons had higher levels of plasma total cholesterol, LDL cholesterol and triglycerides. The plasma concentration of GGT was elevated in male baboons, but no difference between the sexes was observed for albumin.

Table 4.3 shows the heritabilities of liver function markers and cardiovascular risk factors analyzed as part of this study. All were significantly heritable with total cholesterol exhibiting the greatest hertitability. Table 4.4 gives the genetic correlations between plasma GGT, albumin and plasma lipids. No statistically significant association was found between GGT and the cardiovascular related phenotypes. However, negative relationship between plasma albumin and lipids was significant.

The results of a genome wide scan by each chromosome for plasma GGT are displayed in Figure 4.1. A suggestive signal for GGT was observed on the baboon homologue of human chromosome 3 (LOD score 1.95) at marker *D3S1300*. The strongest QTL for GGT was detected between markers *D22S304* and *D22S280* on the baboon homologue of human chromosome 22, at approximately 30 cM with a LOD score of 2.8. This linkage signal is shown in greater detail in Figure 4.2. The maximum LOD score detected in a genome wide linkage scan for albumin was detected on the baboon homologue of human chromosome 10 (Figure 4.3), with a LOD score of 2.3 at 66 cM near marker *D10S1432* (Figure 4.4).

4.5 Discussion

This study has demonstrated that the variation in the plasma levels of GGT and albumin are influenced by genetic factors. The heritability estimates of these liver function markers in baboons are similar to that found in humans. Heritability estimates of GGT were 52% in Australian men and women (Whitefield et al. 2002) and 62% in Danish twins (Bathum et al., 2001), as compared to 55% found in this study. For albumin the additive genetic effect in adult men and women was 36%, which is lower than the value calculated for baboons in this paper (Pankow et al., 2001). This is the first study to publish the QTLs for GGT and albumin in baboons and there is no published information on QTLs for these proteins yet published in humans.

Even though phenotypic correlations between albumin and cardiovascular risk factors have been reported (Schalk et al., 2006; Beddhu et al., 2002; Krijgsman et al., 2002), the evidence of shared genetic effects (i.e. pleiotrophy) between them has been detected for the first time in this paper. The associations found here imply that same set of genes regulate their circulating concentrations.

The most common cause of abnormal liver function is NAFLD, a disease that affects approximately 15-25% of the world's population. The prevalence is higher in the obese and type 2 diabetics (Adams and Angulo, 2005). Patients with NAFLD might be at a higher risk for cardiovascular disease than those without this hepatic condition (Targher G, 2007). The findings of this paper suggest that common genetic factors might be responsible for both of the pathologies.

Gamma glutamyl transferase and albumin are markers of both liver function and oxidative stress. Plasma GGT is a well-established and sensitive marker of hepatic dysfunction.

This enzyme which is present on the cell membrane catalyzes the hydrolysis of extracellular glutathione, a primary cellular antioxidant (Lee et al., 2004). During the metabolism of glutathione by GGT, ferric iron is reduced to the ferrous form. This reaction generates reactive oxygen species that promote the oxidation of LDL cholesterol (Dominici et al., 2005). It is well established that oxidized cholesterol participates in deposition of atherosclerotic plaques. (Meisinger et al., 2006). The circulating concentration of GGT is elevated in CVD and NAFLD patients, (Ruttmann et al., 2006; Marchesini et al., 2005), implying that it might a link between the two diseases. Yet, in this study we did not detect any significant genetic relationship between GGT and cardiovascular risk factors.

In contrast, a significant negative genetic correlation between albumin and cardiovascular risk factors was observed in this study. Traditionally, plasma albumin has been considered to be a good bio-marker of protein malnutrition but now this protein is associated with heart disease (Schalk et al., 2006). The sulphydryl groups (thiols) in albumin scavenge reactive oxygen species and dampen oxidative stress by binding free copper (Cu^{2+}), an ion necessary for the production of free radicals (Don and Kaysen, 2004). In addition, albumin has the ability to bind to free fatty acids, thereby; reducing synthesis of triglycerides and LDL cholesterol. Since the liver manufactures albumin, damage to this organ reduces the blood levels of this protein (Tietge et al., 2004). Lower levels of albumin raises the concentration of unbound free fatty acids that consequently increases the amount of lipid parameters in the blood (Ha et al., 2006).

Insulin resistance and oxidative stress are hypothesized to be the link between fatty liver abnormalities and heart disease (Palomo et al., 2006). Another plausible mechanism associating these two disorders could be abnormal lipoprotein metabolism in individuals with steatotic

liver, i.e. elevated plasma concentrations of triglycerides and low density lipoprotein cholesterol (LDL) (Targher and Arcora, 2006). The high prevalence of cardiovascular disease in NAFLD patients could be due to insulin resistance that results in dyslipidemia (Oretga et al., 2006). It is believed that the increased assembly of very low density lipoproteins (VLDL) in the liver causes hypertriglyceridemia and elevated levels of low density lipoprotein (LDL) (Julius, 2006). Although insulin is known to degrade apolipoprotein B, a constituent of VLDL, this effect is removed in a state of hepatic insulin resistance. Consequently, the triglycerides stored in the steatotic liver are packaged into the VLDL and secreted into the circulation (Taghibiglou et al., 2000). Additionally, insulin resistance is characterized by a reduced efficiency of lipoprotein lipase to clear these particles from the blood, resulting in high levels of LDL since VLDL is metabolized into LDL (Ginsberg, 2006). Finally, the expression of LDL receptors on the liver is downregulated in an insulin resistant liver (Mamo et al., 2001). Ultimately, all of the above increases the plasma levels of atherogenic factors that are known to increase the risk of atherosclerosis.

For GGT, potential positional candidate genes (based upon the homologous region in humans) that are present within the one LOD-support interval of the maximum linkage signal on chromosome 22 include selenoprotein Z (*TRXR2*) (Sun et al., 1999), mitogen- activated protein kinase 1 (*MAPK1*) (Li et al., 1994), macrophage migration inhibitory factor (*MIF*) (Bozza et al., 1995), gamma glutamyltransferase 1 (*GGT1*) and gamma glutamyltransferase 2 (*GGT2*) (Figlewicz et al., 1993). The most promising positional candidate genes within this QTL are the two structural genes that produce the two isoforms (*GGT1* and *GGT2*) of GGT. The genes of interest within the one LOD support interval of the albumin QTL on chromosome 10 are insulin degrading enzyme (*IDE*) (Espinosa et al.,1991), stearoyl –CoA desaturase (*SCD*)

(Zhang et al., 1999), cytochorme P450 subfamily IIC, and polypeptide 9 (*CYP2C9*) (Gray et al., 1995). All of these genes are related to oxidative stress.

In conclusion, this study indicates that a significant genetic component affects circulating levels of GGT and albumin. A common set of genes appear to regulate plasma levels of albumin and cardiovascular related risk factors. These findings support the complex nature of obesity-related comorbidities.

Table 4.1 Relative pairs within the study population

Relationship	Number
Unrelated	3061
Self	350
Parent-offspring	44
Siblings	152
Avuncular	32
Half-siblings	2119
Half avuncular	331
Half 1st cousins	33
Half 1st cousins, 1 rem	15
Half siblings & 1st cousins	3
Half siblings & half 1st cousins	30
Half siblings & half avuncular	5

Table 4.2 Characteristics of phenotypes analyzed in baboons *

Phenotype	Male	Female	p value
Number	96	254	
Age (yrs)	12.90 ± 0.4	15.24 ± 0.3	0.0001
Weight (kg)	31.90 ± 0.6	19.54 ± 0.3	0.0001
Total cholesterol (mg/dl)	91.09 ± 2.59	121.14 ± 2.60	0.0001
LDL cholestrol (mg/dl)	31.53 ± 1.67	53.52 ± 1.83	0.0001
Triglycerides (mg/dl)	46.03 ± 1.77	65.20 ± 1.96	0.0001
γ glutamyl transferase (IU/L)	46.41 ± 1.48	43.43 ± 0.67	0.03
Albumin (g/dl)	4.4 ± 0.05	4.3 ± 0.03	NS

* Mean (s.e.m)

Table 4.3 Heritabilities of liver function markers and cardiovascular risk factors

Phenotype	Heritability ± s.e.m	p value
Total cholesterol	0.68 ± 0.10	< 0.01
LDL cholesterol	0.50 ± 0.10	< 0.01
Triglycerides	0.34 ± 0.11	< 0.01
γ glutamyl transferase	0.55 ± 0.13	<0.0001
Albumin	0.42 ± 0.15	< 0.01

Table 4.4 Genetic (rho_G) correlations between plasma concentration of gamma glutamyl transferase, albumin and cardiovascular risk factors

	Gammaglutamyl Transferase		Albumin	
Phenotype	*ρ Genetic*	*p value*	*ρ Genetic*	*p value*
Total cholesterol	0.18	0.39	-0.60	0.01
LDL cholesterol	0.26	0.35	-0.67	0.01
Triglycerides	-0.41	0.19	-0.86	<0.01

Figure 4.1 String plot of the genome wide scan of plasma concentrations of gamma glutamyl transferase. The y–axis indicates the chromosomal location in cM and the x-axis signifies the LOD score

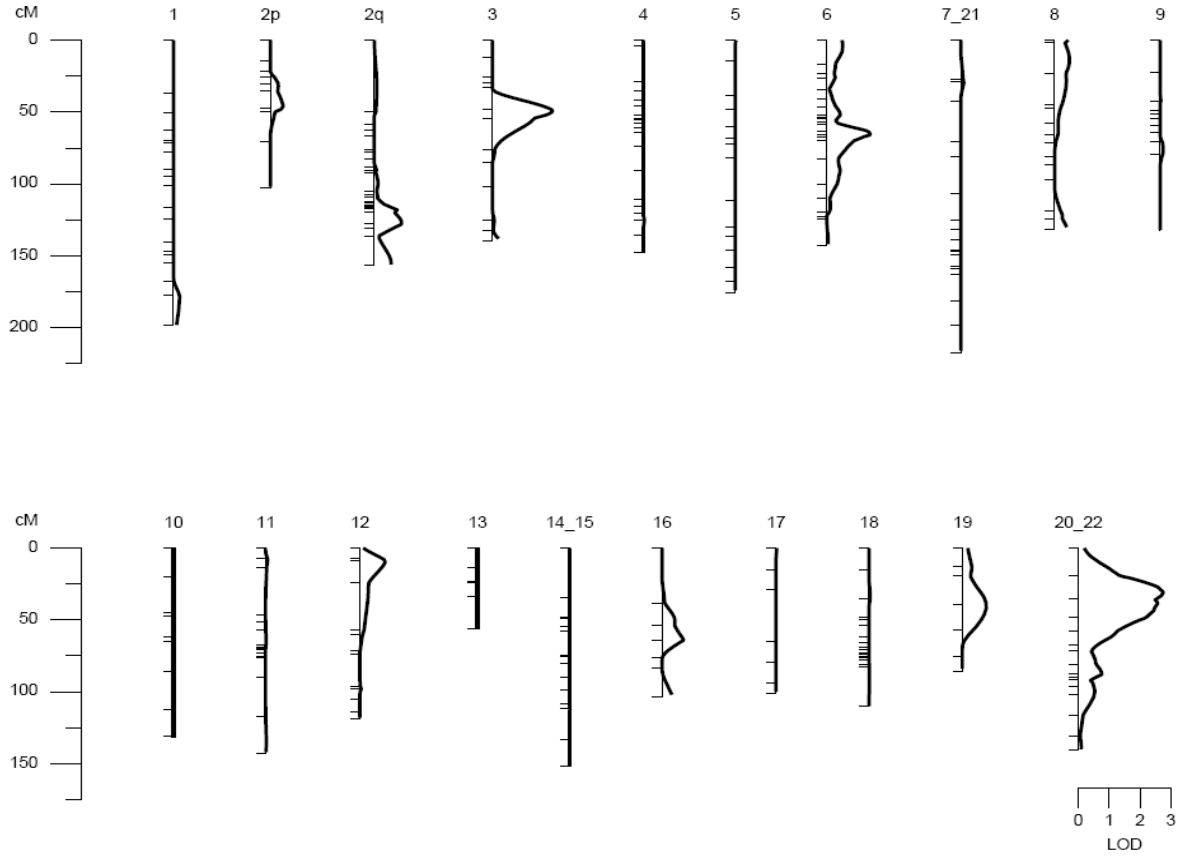


Figure 4.2 LOD plot for plasma gamma glutamyl transferase on chromosome 22

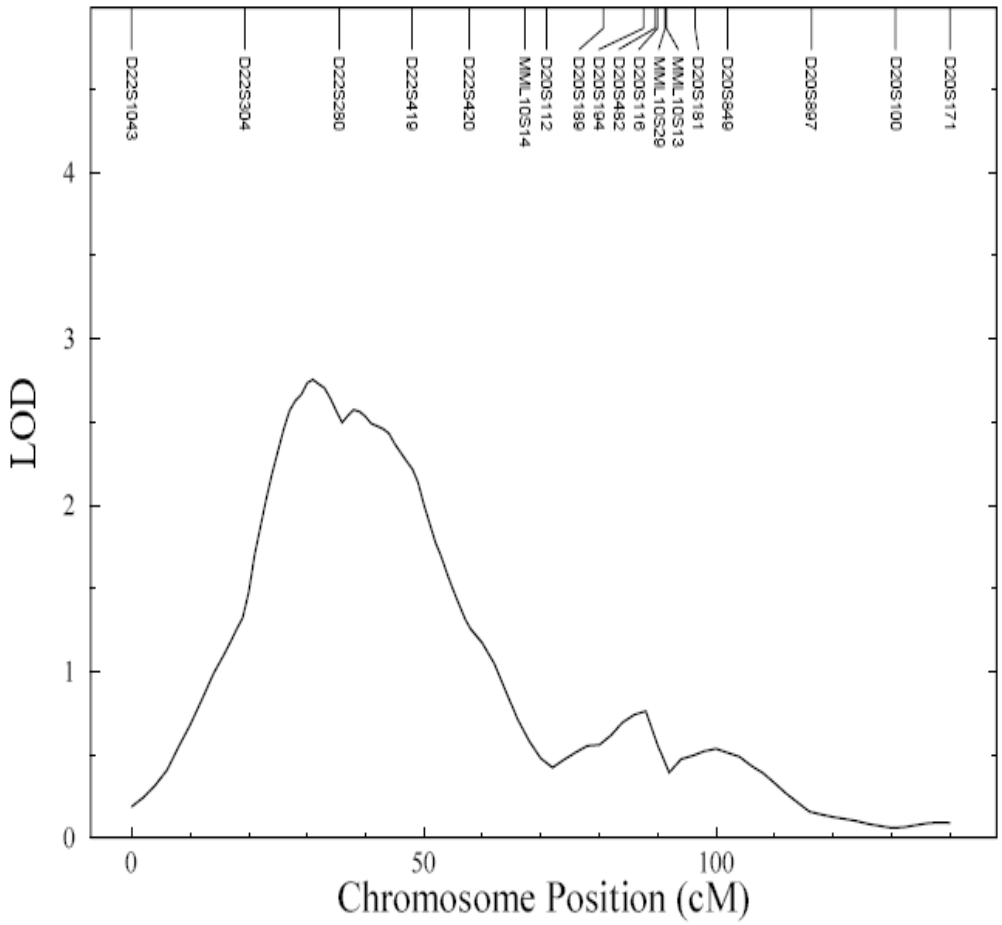


Figure 4.3 String plot of the genome wide scan of plasma concentrations of albumin. The y-axis indicates the chromosomal location in cM and the x-axis signifies the LOD score

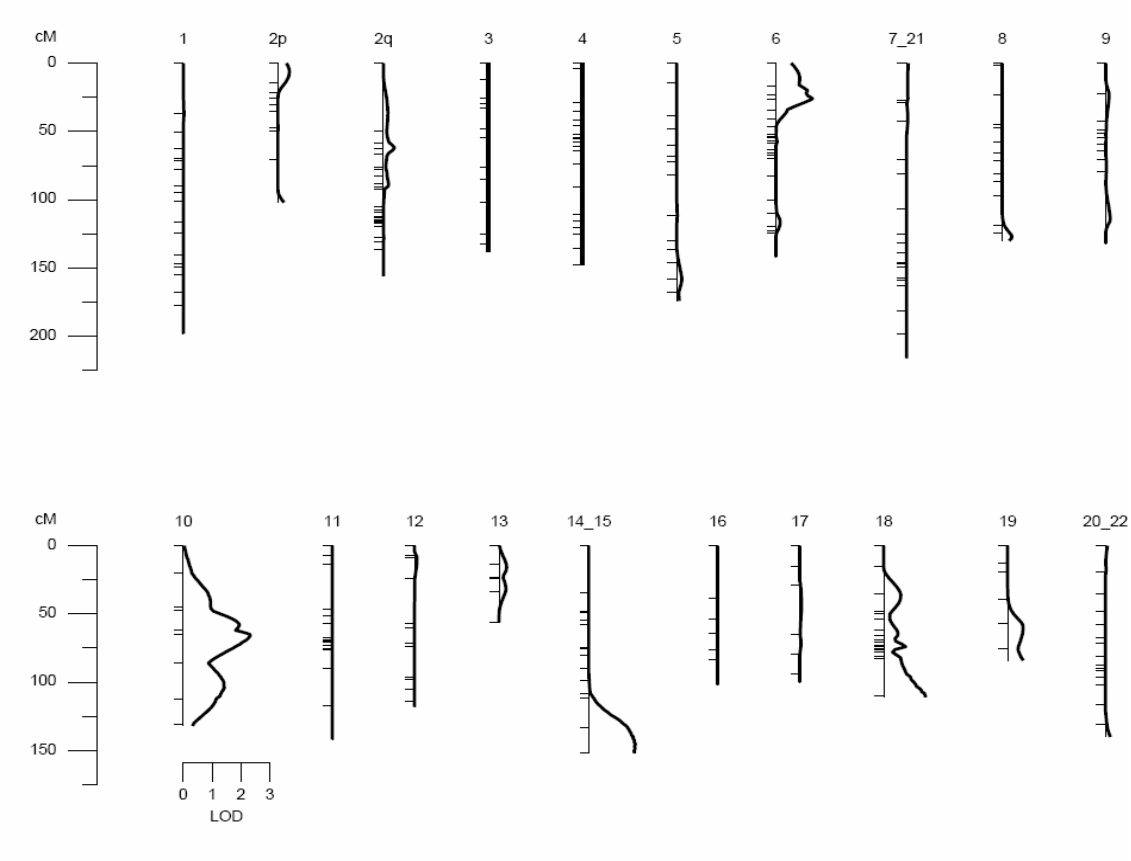
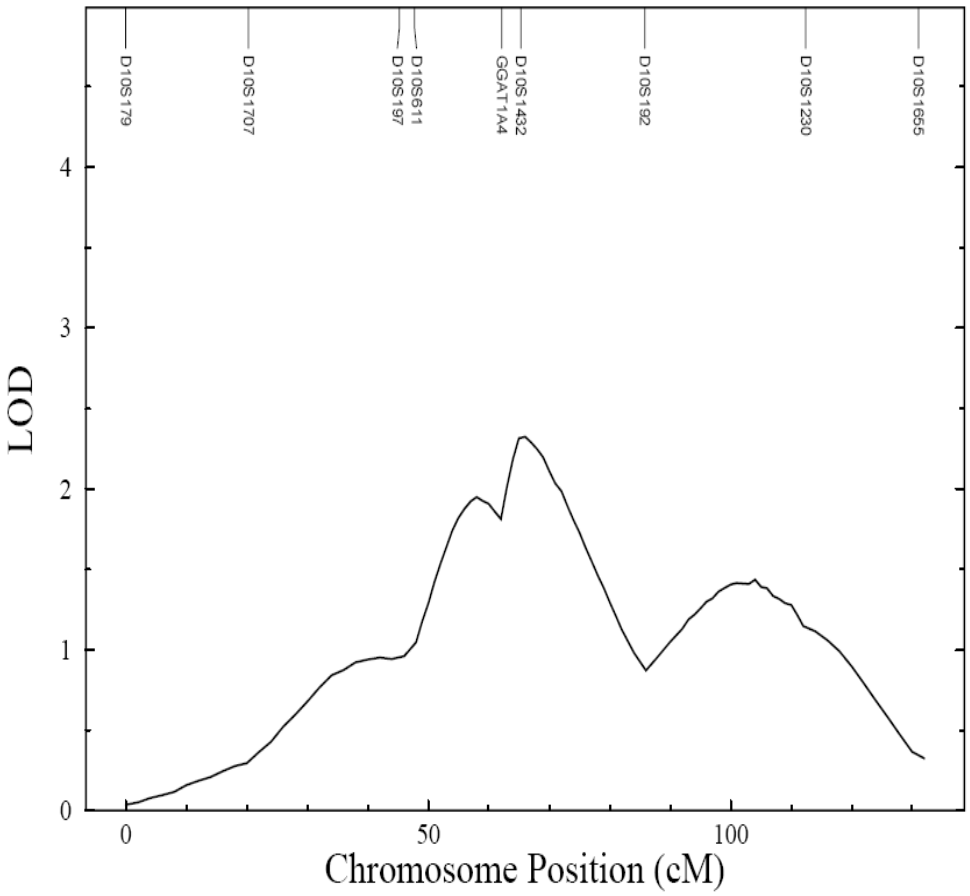


Figure 4.4 LOD plot of plasma albumin on chromosome 10



Chapter 5

Relationship of liver function markers to non-alcoholic fatty liver disease in baboons

5.1 Abstract

Non-alcoholic fatty liver disease (NAFLD) is characterized by hepatic triglyceride accumulation in the absence of excessive alcohol consumption. This disease often coexists with conditions like obesity and insulin resistance. The primary aim of this study was to determine the presence of NAFLD in baboons. The secondary aim was to investigate the association between liver function parameters and various categories of NAFLD.

The study population consisted of 39 baboons housed in a colony maintained at the Southwest Foundation for Biomedical Research (SFBR) in San Antonio, TX, USA. They were fed ad libitum on a standard monkey chow diet (Harlan Teklad 15% monkey diet, 8715, Indianapolis, IN). The animals selected for this project did not have a history of hepatic infection or abnormalities. Fasting plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ glutamyl transferase (GGT) and albumin (ALB), glucose and insulin were assayed by standard laboratory techniques. Hepatic triglyceride was extracted and measured enzymatically. Insulin resistance was calculated by the homeostatic model assessment (HOMA) method. Liver samples were obtained from baboons during necropsy and were stored at -80^{0}C. The liver slides were prepared by standard procedure using thawed liver specimens. The liver specimens were analyzed by two pathologists for histological changes. The findings of this study were inconclusive since the diagnoses of the two pathologists were different from each other. This might be

due to the different grading scales used by each of the histopathologists. Additionally the grading is based somewhat on the subjectivity of each of the pathologists.

5.2 Introduction

Non-alcoholic fatty liver disease (NAFLD) is the primary reason for liver related deaths in the overweight and obese people (Falck-Ytter et al., 2001). This disease initiates with accumulation of triglycerides in the hepatocytes, and eventually progresses to steatohepatitis, fibrosis and cirrhosis (Matteoni et al., 1999). The prevalence of hepatic steatosis and non-alcoholic steatohepatitis (NASH) in the general population is 20-30 % and 2-3%, respectively (Yu and Keeffe, 2002).The incidence of hepatic steatosis is far greater (50-70%) in obese individuals (Wanless and Lentz, 1990).

Important predictors of NAFLD are obesity (Angulo et al., 1999), insulin resistance (Dixon et al., 2001) and dyslipidemia (Chen et al., 2006). It is believed that during these metabolic disturbances, regulation of hormone sensitive lipase by insulin is impaired which increases triglyceride hydrolysis and free fatty acids release from adipocytes (Jaworski et al., 2007). At the same time, insulin resistance also elevates the circulating levels of glucose and insulin (Stump et al., 2006). Higher uptake of free fatty acids by the liver and up regulation of hepatic lipogenic genes by insulin elevates the triglyceride pool in the liver. Insulin also promotes the reesterification of fatty acids rather than oxidation (Diraison et al., 2003), in addition to impeding the export of triglycerides (Gibbons et al., 2002) from the liver. Moreover, import of unesterified fatty acids to the hepatocytes may surpass the mitochondrial oxidation capacity and, ultimately, favor triglyceride accumulation (Vendemiale et al., 2001). This fat deposition

in the liver exposes the liver cells to further injury from free radical species which results in non-alcoholic fatty liver disease.

In lipid-laden liver free fatty acids induce the expression of genes involved in fatty acid oxidation in the mitochondria, as well as peroxisomes or microsomes, via stimulation of the peroxisomal proliferator-activated receptor-α (Jansen P, 2004). However, reactive oxygen radicals are generated during the oxidation of unesterified free fatty acids (Natarajan et al., 2006). Consequently, oxidative stress results in neutrophil infiltration, leading to steatohepatitis or inflammation.

Liver biopsy is the only method that can truly distinguish between the different stages of the NAFLD (Hubscher S, 2004). However, due to cost and risk associated with biopsy it is not feasible to perform this procedure for all patients suspected to have NAFLD. Non-invasive methods such as ultrasound (Joy et al., 2003), computer tomography (Limanond et al., 2004), and magnetic resonance imaging (Saadeh et al., 2002) are ideal for measuring the extent of liver damage due to steatosis. Liver injury markers (Schindhelm et al., 2006) are the cheapest and most convenient method used widely to diagnose liver dysfunction; but these are not the most reliable indicator of the status of fatty liver disease. None of these techniques surpass the sensitivity and specificity as great as of liver biopsy (Sanyal A, 2002).

Some of the baboons maintained in the colony at the Southwest National Primate Research Center are known to develop obesity (10%) and insulin resistance (4%), in spite of similar diet and environmental conditions. Therefore, these animals are an excellent model to study obesity-related diseases (Tejero et al., 2004). The goal of this project is to

explore the occurrence of non-alcoholic fatty liver disease and to determine its relationship with markers of liver injury in baboons.

5.3 Materials and methods

Animals

The study population consisted of 39 (18 males, 21 females) baboons (Papio hamadrayas anubis). These animals were gang-housed in a colony maintained at the Southwest Foundation for Biomedical Research (SFBR) in San Antonio, TX, USA. They were fed ad libitum on a low fat standard monkey chow diet (Harlan Teklad 15% monkey diet, 8715, Indianapolis, IN). The animals selected for this project did not have a history of hepatic infection or abnormalities.

Sampling and assays

All procedures performed were approved by the Institutional Animal Care and Use Committee of the SFBR. Blood samples were collected following an overnight fast (12 hours) from the antecubital vein of animals sedated with ketamine prior to necropsy. The samples were centrifuged at 2000 x g for 10 minutes and the plasma was stored in aliquots at -80^0C for future analyses. The weights of the animals were measured on a calibrated electronic scale (GSE, Chicago, IL). Liver proteins were assayed by standard laboratory techniques using an Alfa Wasserman ACE clinical chemistry instrument (West Cladwell, NJ). Hepatic triglycerides were extracted by the method described by Folch et al. (1957). Triglyceride content of the tissues was measured enzymatically, using a commercial reagent (Stanbio Laboratory, Boerne TX), in a spectrophotometer at 550 nm. Glucose was analyzed by the glucose oxidase method on an Analox spectrophotometer (Tejero et al., 2004). Insulin was measured by chemiluminescence in a Luminex 100 with

endocrine multiplex immunoassay (Linco Research, Inc). Insulin resistance was calculated by the homeostatic model assessment (HOMA) method (Matthews et al., 1985). Samples whose replicates had >5% variations were reanalyzed.

Histology

Liver samples were obtained from baboons during necropsy. These liver specimens were immediately frozen by dipping in liquid nitrogen. To prepare liver slides the tissues were thawed and fixed in formalin, embedded in paraffin to prepare 5 μm sections via microtome (Olympus America, Melville, NY), and then stained with hematoxilin and eosin (Maislos et al., 2005). The liver specimens were graded by two pathologists. The first pathologist (NU) used the Brunt et al. (1999) grading protocol and the second pathologist (ED) used the grading scheme proposed by Kleiner et al (2005). To compare the results of the two pathologists the animals were grouped into two categories. Category 1 contained baboons with either absent or mild steatosis. Baboons with steatosis, coupled with lobular inflammation and ballooning, were included in category 2.

Statistical methods

Descriptive statistics and other statistical analysis were performed using SPSS (SPSS Inc., Chicago, IL; V 10.0). Results are expressed as mean (95% confidence interval). Student's tests were performed to determine the difference in the continuous variables between the two categories. Pearson chi-square test was performed to compare the results of the two pathologists (Kleiner et al., 2005).

5.4 Results

The two categories created in this study to compare the diagnosis of the two pathologists are described in Table 5.1. Table 5.2 shows the number of cases diagnosed as category 1 and 2 NAFLD by the two pathologists. The findings of each of the pathologists are elaborated in Table 5.3. There were no significant differences between the diagnoses of the two pathologists for steatosis, ballooning or portal inflammation. However, the results for lobular inflammation varied substantially between the two pathologists. Descriptive statistics of baboons with NAFLD, based on the diagnosis of the first pathologist, are shown in Table 5.4. The ages of the animals in the two groups did not differ significantly. The mean weights of the animals in category 2 NAFLD were higher, but not significantly, than that of the baboons in category 1. According to the grouping for NAFLD by the first pathologist, plasma levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma glutamyl transferase, hepatic triglyceride content and insulin sensitivity measured by the homeostasis model assessment method (HOMA) did not differ statistically between the two groups. However, serum albumin was significantly lower for the baboons with category 2 NAFLD.

Descriptive statistics of baboons with NAFLD based on the diagnosis of the second pathologist are shown in Table 5.5. In this distribution animals diagnosed with NAFLD were older than the baboons with absent or mild steatosis. Weights, and plasma levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma glutamyl transferase, albumin, hepatic triglyceride and insulin sensitivity did not differ statistically between the two groups.

Figure 5.1 show a healthy baboon liver. Figure 5.2 illustrates the presence of mild steatosis in baboon liver (Category 1). Figure 5.3 shows advanced stage of the disease, i.e., steatosis coupled with inflammation and ballooning (Category 2).

5.5 Discussion

This is the first study to investigate the presence of non-alcoholic fatty liver disease in baboons and to explore its relationship with liver proteins. Other animal models used for the study of fatty liver disease are usually subjected to dietary intervention or genetic manipulations (Zou et al., 2006; Ito et al.; 2006; Kainuma et al., 2006; Shiri-Sverdlov et al., 2005). The uniqueness of this project was the use of captive baboons, with a potential to develop spontaneous obesity and insulin resistance that could demonstrate the presence of obesity-related hepatic pathology.

The occurrence of NAFLD was not affected by the sex of the animals. Based on earlier human studies it was believed that this condition affected women more than men (Powell et al., 1990; Diehl et al., 1988; Lee RG, 1989). Current evidence suggests that NAFLD is observed equally in both the sexes (Bacon et al., 1994; Teli et al., 1995). The presence of NAFLD has been reported in all age groups. However it is more common in older patients, especially those 50 years and older (Patton et al., 2006; Nomura et al., 1988; Adam et al., 2005). In the present study, based on the findings of the first pathologist, no significant difference was found between the ages of the animals in the two diagnostic groups. However, based on the findings of the second pathologist, the baboons with category 2 NAFLD were significantly older than the baboons with category 1 NAFLD. This disparity in the results might be due to sample size in each of the groups based on the diagnosis of the pathologists.

It is well known that the prevalence of fatty liver disease increases with body weight (Angulo et al., 1999; Bellentani et al., 2000). It has been documented that compared to 10-15% of the lean subjects the occurrence of this hepatic condition is observed in about 70-80% of the obese individuals (Sanyal A., 2002). However, in this study only the baboons diagnosed as category 2 NAFLD by the first pathologist were found to have higher body weight than their counterparts assigned to category 1 NAFLD. Based on the results of the second pathologist, significant differences between the weights of the animals in each group were not seen. This might be due to various reasons such as starvation leading to rapid weight loss (Whitehead, 1980) or other unidentified physiological or genetic factors which might present NAFLD like histological changes without significant weight gain.

Interestingly, plasma levels of AST, ALT and GGT in the lean baboons were above the cut-off point value (30 IU/L) used for humans (Prati et al., 2002). Although no substantial differences in the concentrations of liver functions markers were observed between lean and obese, the levels of ALB were significantly lower in obese baboons, as compared to lean. Moreover, as seen in humans, the levels of ALT were higher than that of AST (Clark J, 2006) in baboons. A notable observation of this research is that the circulating amounts of the liver proteins did not vary with the severity of the histological lesions. Liver damage biomarkers are known to not correlate with the progression of the NAFLD. This is why a liver biopsy is necessary to make an accurate diagnosis of the stage of the fatty liver disease (Mofrad et al., 2003). Nonetheless, due to the cost and risk involved with biopsies, biochemical indicators are still the preferred medium to gauge the prevalence of NAFLD in the population (Ruhl and Everhart, 2003; Clark et al.2003).

Insulin resistance has been implicated in the pathogenesis of NASH (Angulo , 2002). In this sample of baboons, we did not find a substantial increase in HOMA, a parameter for insulin resistance, between the healthy baboons and those diagnosed with NAFLD. This might be due to the dissimilar results between the two pathologists. However, the circulating levels of insulin and insulin resistance index (HOMA) values are known to be high in the obese baboons. In this study all the baboons had high HOMA values ≥ 3 (Tejero et al., 2004).

Histological diagnosis of NAFLD was the key feature of this study. Although different grading systems were used by the pathologists, some of the histological scores were combined for the purpose of comparison and discussion. In this study the presence of mild steatosis is assumed to be benign and not a diseased state (Matteoni et al. 1999). Thus, the two categories are category 1 which includes baboons with absent or mild steatosis. Whereas, baboons in category 2 are diagnosed as NAFLD due to the presence of steatosis that is coupled with inflammation and ballooning. Steatosis was commonly found in the liver slides of baboons. In this type of steatosis a single large droplet of fat occupied the whole cytoplasm displacing the nucleus (Brunt and Tiniakos, 2002). This intra-cytoplasmic fat accumulation might occur due to dysregulated import, manufacture and export of lipids to the liver (Angulo, 2002). According to the grading system used by the first pathologist, steatosis was graded on a scale of 0-3. The scores were given to each slides based on the extent of cells that were steatotic, i.e., 0 = none, 1= up to 33%, 2 = 33-66% and 3 = > 66% (Brunt et al., 1999). However, the second pathologist graded for steatosis using a different scale. The presence of less than 5% of steatotic cells was given a score of 0, 5-33% = 1, > 33-66% = 2, and > 66% = 3 (Kleiner et al., 2005). This

scoring was no doubt influenced by the subjective nature of these grading scales, resulting in wide variation in the diagnoses of the two pathologists. At this point it is still unknown which pathologist was accurate in their interpretation of the slides.

In NAFLD, inflammation in the lobules (lobular inflammation) and/or in portal tracts (portal inflammation) are known to occur. The latter is usually non-existent or mild (Brunt, 2001). A key characteristic of lobular inflammation is presence of mixed immune cells such as lymphocytes, macrophages, Kupffer cells and mostly polymorphonuclear leukocytes (PMN) (Brunt, 2004). The presence of portal inflammation is not necessary for the diagnosis of NASH (Matteoni et al. 1999). Portal inflammation was graded by the first pathologist on a scale of 0 – 3, 0 for no portal inflammation, 1 for mild, 2 for moderate and 3 for severe. On the other hand the second pathologist gave the slides a score of 0 for none to minimal portal inflammation and 1 for greater than minimal portal inflammation. In this case it is very difficult to judge the threshold for minimal inflammatory changes in the portal area. Moreover, these grades might be affected by the personal bias of the respective pathologist leading to differences in diagnoses of the cases. For the grades for lobular inflammation, however, the pathologists used the same scale. According to both, a score of 1 was given when 1- 2 inflammatory foci were seen at 20X magnification, 2 for the presence of 2-4 foci and 3 for more than 4 foci. In spite of the same scale for lobular inflammation, variation was observed in the diagnosis between the two pathologists. This most likely reflects the subjective nature of the grading system.

Ballooned or swelled hepatocytes have clear cytoplasm. Mallory's hyaline formed from the cytoplasmic fragments is present due to severe cell injury (Hubscher, 2006). However, the mallory hyaline may or may not be seen. Therefore, the presence of

Mallory hyaline is not important for the diagnosis of steatohepatitis (Brunt, 2005). Ballooning normally affects liver cells in acinar zone 3 and is one of the most distinctive traits of the more progressed form of the fatty liver disease (Matteoni et al., 1999). According to the Brunt grading system (Brunt et al., 1999) used by the first pathologist, ballooning is graded based on the location and extent of affected cells. However, the Kleiner grading system (Kleiner et al., 2005) gives a score of either 1 or 2 based on the estimate of severity. The scores of the two pathologists were comparable for ballooning. However no statistical test could be performed to verify the inter observer agreement since the two grading scales were different.

In summary, significant associations between weight and insulin resistance and occurrence of NAFLD in baboons could not be established. This lack of significance is most likely due to disparity in the diagnoses of the two pathologists and/or sample size. Although baboons present the clinical and histological features of NAFLD similar to that seen in humans, future investigations with a much larger sample size will be required to capture the effect of weight, insulin resistance and dyslipidemia on the occurrence of fatty liver disease. This animal model may be helpful in understanding the underlying pathogenesis NAFLD since common environmental confounders such as alcohol consumption associated with human studies is not a problem. A secondary objective of subsequent research should be to validate the use of the liver biomarkers as a useful noninvasive tool for assessing NAFLD related liver damage in baboons. Future study should be done using liver slides made from fresh tissue samples. In this study some of the results may be artifacts due to the processing of frozen liver specimens. Finally, future projects should take into account the subjective nature of most grading systems

and use only one grading scale. The slides should be graded by the same pathologist twice, with the pathologist blind to the samples each time.

In conclusion, this study provides evidence of occurrence of histological lesions similar to that seen in humans suffering from NAFLD. However, these histological changes could not be associated to obesity and insulin resistance in this project.

TABLE 5.1 Grading system for non-alcoholic fatty liver disease (NAFLD)

Categories of NAFLD	Definition
Category1	Absent and/or mild steatosis
Category 2	Steatosis coupled with inflammation and ballooning

TABLE 5.2 Rating of non-alcoholic fatty liver disease as diagnosed by the two pathologists

	Rating of liver biopsies	
	Category1	Category 2
Pathologist 1 (NU)	35	4
Pathologist 2 (ED)	18	21

Table 5.3 Histological features of non-alcoholic fatty liver disease observed in baboons by the two pathologists

Histological Features	Pathologist 1 N = 39	Pathologist 2 N = 39	p value*
Steatosis			
< 33%	36	10	
33-66%	2	20	0.70
> 66%	1	9	
Ballooning			
None	33	37	
Few ballooned cells[a]	5	2	0.83
Many ballooned cells[a]	1	N/A	
Portal inflammation			
None to minimal	22	30	0.11
Greater than minimal	17	9	
Lobular inflammation			
<2 foci per 20X field	27	13	
2-4 foci per 20X field	10	26	0.01
> 4 foci per 20X field	2	N/A	

*Pearson chi- square test between groups
[a]Brunt et al., 1999

Table 5.4 Descriptive statistics of baboons with Non-alcoholic fatty liver disease based on the diagnosis of the first pathologist

	Category 1 N = 35	Category 2 N = 4	p value
Gender			
Male	15	3	
Female	20	1	
Age (yrs)	15.7 (13.4,17.9)*	21.7 (18.4,24.9)	0.10
Weight (kg)	22.8(19.4,26.1)	34.9 (23.6,51.8)	0.06
Alanine aminotransferase (IU/L)	47.5 (39.5, 57.3)	43.2 (34.5, 54.0)	0.74
Aspartate aminotransferase (IU/L)	35.7 (30.4,42.0)	33.4 (28.4, 39.3)	0.79
Gamma glutamyl transferase (IU/L)	49.1 (44.9,53.7)	42.6 (38.1,47.5)	0.08
Albumin (g/dl)	4.6(4.4,4.9)	3.7(3.1,4.4)	0.009
Hepatic Triglyceride (mg/g)	253.7 (232.7, 276.6)	260.7 (236.7, 287.2)	0.69
HOMA[a]	3.4 (2.4,4.9)	5.6 (3.6, 8.8)	0.13

*Mean (95 % confidence interval)
[a]Homeostasis model assessment method

Table 5.5 Descriptive statistics of baboons with non-alcoholic fatty liver disease based on the diagnosis of the second pathologist

	Category 1 N = 18	Category 2 N = 21	p value
Males	7	11	
Females	11	10	
Age (yrs)	13 (10, 15.9)*	19.1 (16.6, 21.6)	0.003
Weight (kg)	24.1 (19.9, 29.1)	23.6 (19.4, 28.6)	0.88
Alanine aminotransferase (IU/L)	46.4 (35.9, 59.9)	47.7 (37.9, 59.9)	0.88
Aspartate aminotransferase (IU/L)	35.5 (26.3, 47.8)	35.5 (32.1, 39.3)	0.99
Gamma glutamyl transferase (IU/L)	46.4 (40.7, 53)	50.2 (45.3, 55.6)	0.36
Albumin (g/dl)	4.7 (4.4, 5.1)	4.4 (4.1, 4.7)	0.13
Hepatic Triglyceride content(mg/g)	266.4 (241.5, 293.9)	244.1 (216.9, 274.6)	0.28
HOMA[a]	4.4 (2.7, 7.2)	3.0 (1.9, 4.7)	0.27

*Mean (95 % confidence interval)
[a]Homeostasis model assessment method

Figure 5.1 Healthy baboon liver (hematoxylin and eosin 40X)

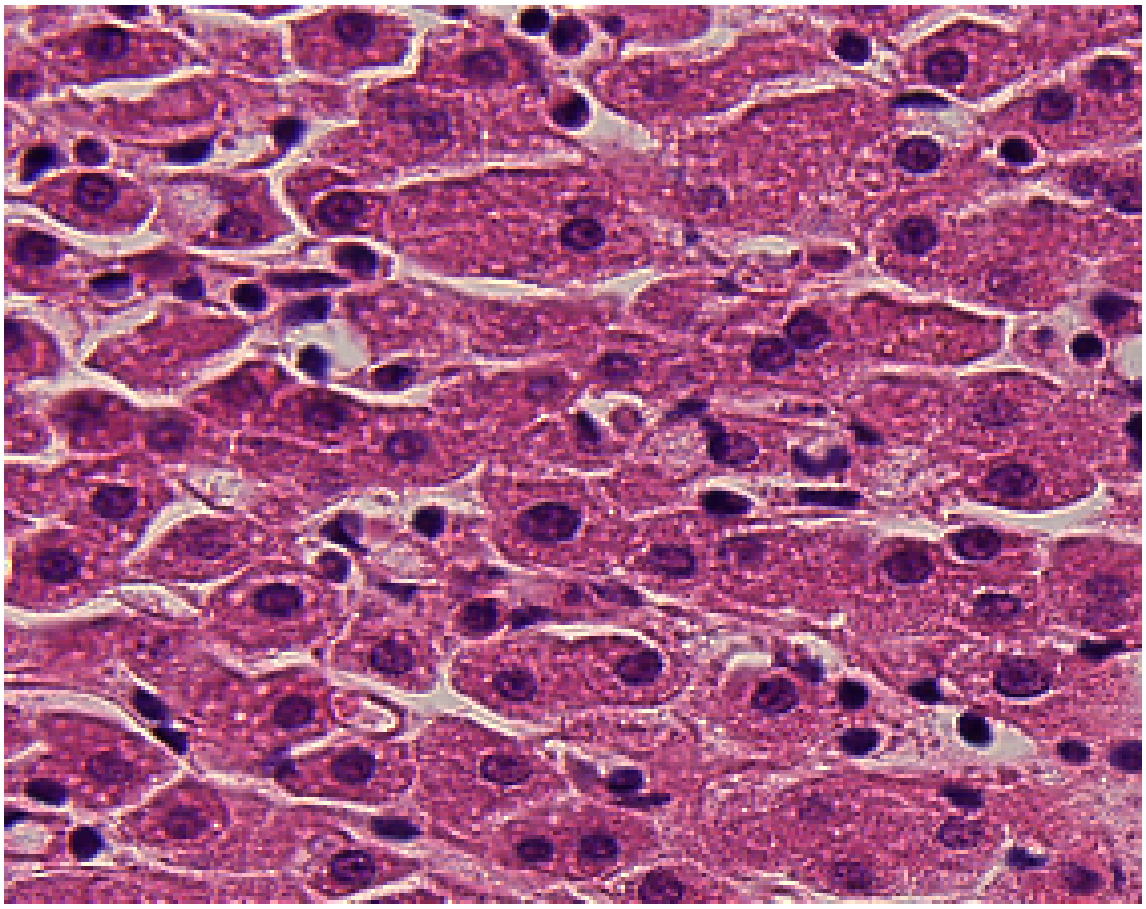


Figure 5.2 Category 1 of non-alcoholic fatty liver disease characterized by the presence of macrovesicular steatosis (hematoxylin and eosin 20X)

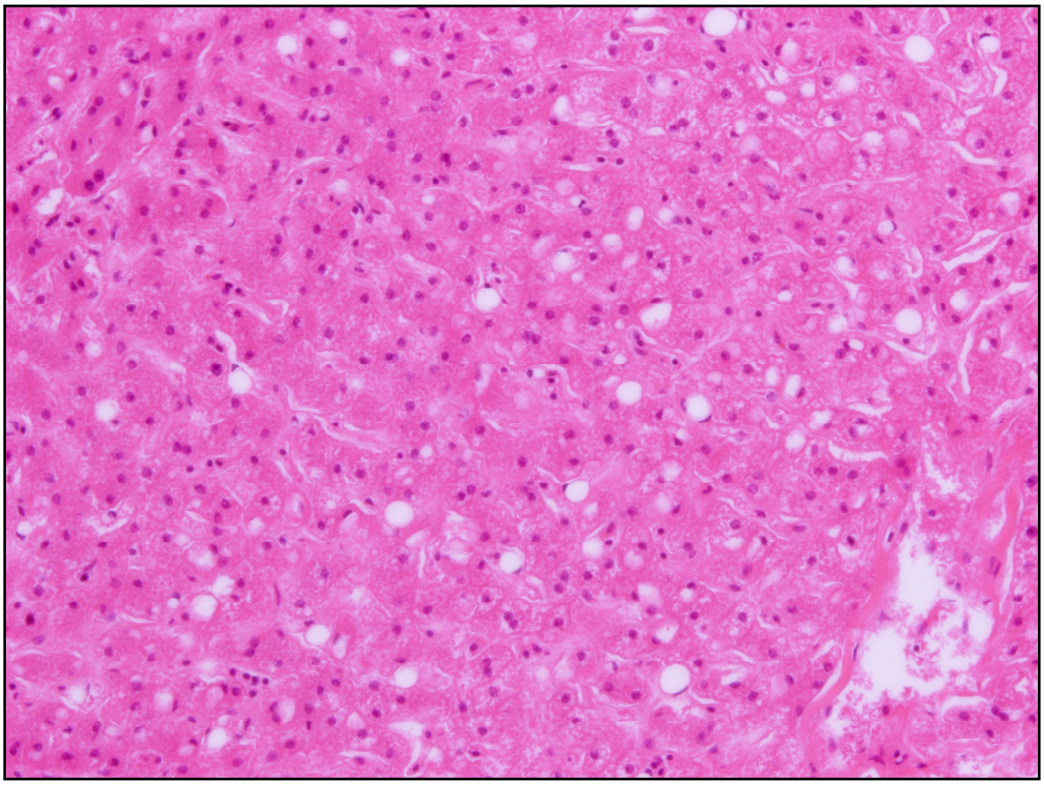
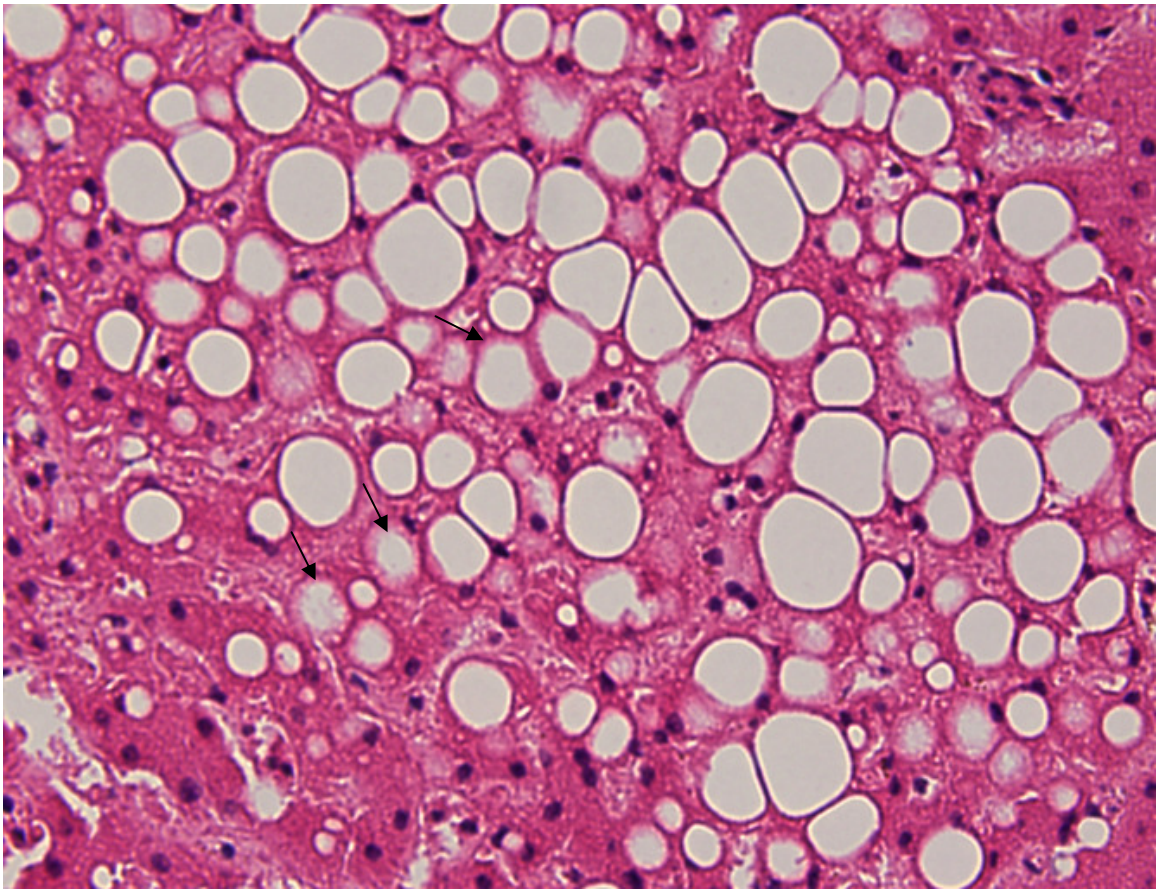


Figure 5.3 Category 2 of non-alcoholic fatty liver disease characterized by presence of steatosis, inflammation and ballooning (hematoxylin and eosin 40X)



Arrows shows the ballooned hepatocytes

Chapter 6

Conclusions and Recommendations

This study shows the relationship between surrogate markers of non-alcoholic fatty liver (NAFLD) and adiposity related factors in baboons. The findings of this project suggest that reduced number of mature adipocytes in combination with inflammation may be involved in the pathogenesis of fatty liver disease associated with obesity and insulin resistance. A strong genetic influence on the adipocyte volume and number and circulating levels of liver function markers was observed in this project. A significant genetic correlation between liver proteins and adiposity and cardiovascular associated factors was also established. No significant relationship between the variations in liver function markers and various stages of NAFLD was found. Histological changes were observed independent of weight or insulin resistance status of the animals.

Results from chapter 2 illustrate the association between circulating concentrations of MCP-1, adipocyte number, insulin resistance index (HOMA) and plasma levels of liver injury proteins. These relationships further strengthen the hypothesis that obesity related inflammation leads to reduced insulin sensitivity, which ultimately leads to fat deposition in the liver and raises the levels of liver related enzymes. Based on these findings the next step would be to explore the mechanism involved in the adipocyte dedifferentiation since very little research has been done in this area. Subsequent studies with a larger sample size should try to ascertain an association between insulin resistance and markers of fatty liver disease since impaired insulin

sensitivity is believed to the basis of this hepatic pathology. The drawbacks of this study were the use of a small sample and also lack of histological diagnosis of NAFLD using liver biopsies to establish the prevalence of hepatic lesions in these animals.

In chapter 3 a substantial heritability for aspartate aminotransferase, a liver enzyme and omental adipocyte volume and number was estimated. A strong signal for fat cell volume (LOD score = 3.2) was located on baboon homologue of human chromosome 6 at 73cM. The occurrence of linkage in this chromosomal region is of value since localization of other obesity related phenotypes in humans such as birth weight (Arya et al., 2006), body mass index (Atwood et al., 2006) and sum of skin folds (Duggirala et al., 2001) also has been found on chromosome 6q.

For the first time a significant genetic correlation between the physical characteristics of adipocytes and a biomarker of hepatic dysfunction was detected. These findings bolster the concept that reduced number of mature adipocytes could be responsible for the pathogenesis of co-morbitites related with increased adiposity. A future direction would be to identify the common set of genes and the pathways, which might influence fat cell volume and number and NAFLD.

In this study we could not identify the QTLs for adipocyte number and AST. This might be due to the sample size which reduces the power to detect the loci for a trait of interest. Therefore this study should be done again with a substantially large number of baboons to obtain a significant QTL for adipocyte number and AST.

Chapter 4 elucidates the results of the genome wide scan for plasma concentrations of γ glutamyl transferase (GGT) and albumin (ALB). A linkage for GGT was located on baboon homologue of human chromosome 22 (LOD = 2.8). A QTL (LOD

=2.3) was detected on the baboon homologue of human chromosome 10 for ALB. These findings are notable, as other phenotypes that are linked with obesity also have been mapped to these chromosomes. For example, peaks for genes expressed in human visceral adipose tissue (Yang et al., 2002), percentage fat mass (Dong et al, 2003)) and BMI of moderately obese individuals (Bell et al., 2004) were found on chromosome 22 and 10, respectively. Future research efforts should involve fine mapping the identified genome segments. The functional gene for GGT resides on chorosome 22 which may be sequenced and single nucleotide polymorphisms (SNPs) may be typed within this gene. These endeavors would help us to evaluate the affect of variation in this gene on obesity associated phenotypes.

This study found significant genetic correlations between plasma albumin and cardiovascular risk factors such as total cholesterol, LDL cholesterol and triglycerides. These results suggest that a common pathogenic agent, plausibly oxidative stress, might be responsible for the occurrence of both the liver and heart dysfunction in the state of obesity. This project failed to identify a relationship between the GGT and cardiovascular related phenotypes. One of the reasons for this could be due to sample size, which limits the power of association. A larger sample may allow the detection of a significant correlation between these traits and aid in detection of a significant LOD score for genome wide scan using bivariate analyses.

A major limitation of these first three studies is the unavailability of liver biopsies to determine the exact stage of NAFLD in the animals. In addition no other anthropometric measurements other than body weight were available to verify the degree of adiposity in baboons. Future studies should include information about percent body fat

for a more accurate estimate of weight gain. Another drawback of this study is the possibility of liver enzymes elevation due to undiagnosed pathological conditions.

In chapter 5 associations of liver injury biomarkers with the severity of the disease in baboons was found to be conflicting between the reports of the two pathologists. Although the plasma levels of liver proteins increase in the event of a hepatic injury (Angulo P, 2002), there are reports that NAFLD may occur in patients, in spite of normal levels of liver damage indicators (Sanyal A, 2002). This discrepancy suggests that liver function markers are not the most reliable indicator of the extent of liver damage due to fatty liver disease.

The major drawback of this study was the use of frozen liver tissues for the preparation of slides. Processing steps such as thawing and fixing old specimens may have compromised their structural integrity. The handling procedure may have created artifacts which could have affected the accuracy of the diagnosis. An ideal experiment would have been to include age- and sex-matched lean and obese baboons in the study. In this study there were more lean baboons than obese ones. Most importantly, two different pathologists graded the liver slides and there was no similarity between their diagnoses. This might be due to the differences in their area of expertise. The first pathologist was a practicing human medical doctor in Mexico City; whereas, the second pathologist was a veterinarian at Southwest Foundation for Biomedical Research in San Antonio, Texas.

Body weight and insulin resistance did not influence the incidence of NAFLD in this group of animals. This might be due to the limited sample size and the dissimilar results of the two pathologists, which might have reduced the capacity to estimate significant relationships. However no trends exist. Although we found the prevalence of

NAFLD in lean baboons, it could be due to many reasons such as hepatic iron overload, starvation leading to rapid weight loss, and/or jejunal diverticulosis (Angulo, 2002). In this project presence of hepatitis C was the only exclusion criteria used. Future studies should employ more stringent screening protocols such as animals with history of rapid weight loss, hepatic iron overload or other gastrointestinal diseases should be excluded from the cohort. However, it is not believed that our baboons exhibited any of these problems.

It is important to investigate the pathophysiology relating obesity, insulin resistance and inflammation and NAFLD in the primate. Future research efforts should focus on the exploring the contribution of oxidative stress to both fatty liver and cardiovascular diseases. It is also important to identify loci that affect the obesity-related traits and liver proteins. Subsequent endeavors must be concentrated on bivariate genome wide scans to identify the pleiotropic genes that affect the phenotype of interest.

In summary, objective 1 identified that plasma levels of monocyte chemoattractant protein -1 are associated with adipocyte volume and number, HOMA and circulating levels of liver function markers in baboons. Objective 2 established that omental adipocyte volume and number and plasma levels of aspartate aminotransferase are highly heritable traits in baboons. A significant correlation between aspartate aminotransferase and adipocyte volume and number also was determined. Objective 3 found a quantitative trait locus (QTLs) for circulating levels of γ glutamyl transferase and albumin along with strong bivariate relationships between albumin and cardiovascular-related risk factors. However, objective 4 could not establish a conclusive influence of body weight and insulin resistance on the occurrence of NAFLD in baboons.

In conclusion, these investigations initially suggested that obesity related factors might lead to an elevation of liver function markers in baboons. These results of this dissertation implied that baboons could be used to study the pathology related to elevation of liver proteins due to obesity and insulin resistance. In addition, this animal may be a model for genetic studies due to the lack of environmental confounders present in human studies. Liver biopsy is the best tool to diagnose NAFLD in baboons since the whole spectrum of the disease may occur in spite of normal levels of liver biomarkers. However, in this study we used frozen liver samples obtained during necropsy rather than biopsy. Nonetheless the results obtained using frozen liver samples did not match the theory. Thus, we can not conclude that liver function markers are a good indicator of NAFLD or that NAFLD is altered in obesity.

Appendix A

Quantitative Genetic Analysis

This project has employed a maximum likelihood-based variance decomposition approach implemented in the computer program sequential oligogenic linkage analysis routinues (SOLAR) to conduct genetic analyses (Almasy& Blangero, 1998).Univariate analysis was conducted to determine the heritability

Quantitative genetic analysis estimates the effect of heritability on the variance of a trait (Comuzzie et al., 2001). This procedure is used to identify specific genes whose influence on a phenotype is quantifiable. It bifurcates total phenotypic variance in a trait (σ_P^2) into genetic (σ_G^2) and environmental (σ_E^2)constituents.

$$\sigma_P^2 = \sigma_G^2 + \sigma_E^2$$

The genetic element of the above equation can be divided into additive genetic effects (σ_A^2), dominance (σ_D^2), and epistasis {gene-gene interaction} (σ_I^2). The second component, the environmental element, is further segregated into assessable fractions such as housing, physical activity and immeasurable fractions (Falconer &Mackay, 1996).

The additive genetic effect is genetic variance due to the summation of the influence of all individual alleles that affect a trait. Dominance is defined as the genetic variance at a single locus due to dominance of one allele over another. Epistasis is when the phenotypic outcome of alleles of one gene is masked by alleles of another gene (Griffiths et al., 2000).

The heritability (h^2) of a trait is defined in two ways:

1) The proportion of phenotypic variance due to all genetic effects (additive, dominance and epistasis)

$$h^2 = \sigma_G^2 / \sigma_P^2$$

2) The proportion of phenotypic variance exclusively due to additive genetic effect.

$$h^2 = \sigma_A^{2/} \sigma_P^2$$

Heritability is derived from a likelihood ratio test. This test compares the likelihood of a model whose heritability is estimated with the likelihood of a model whose heritability is confined to zero. Twice the difference of the logarithmic likelihood is distributed asymptotically as a ½: ½ mixture of a chi-square variable with one degree of freedom and a point mass at zero (Self & Liang, 1987).

Genome wide scan is a method that identifies genes that may influence a trait. This approach does not speculate about the relevance of a gene or the chromosomal region; instead it searches for variation over the entire genome (Comuzzie et al., 2001). The genome scan output validates a candidate chromosomal region and/or a positional candidate gene (Almasy et al., 1999). A positional candidate gene is deemed important only if it is close to a QTL. A QTL detected through linkage analysis while conducting a genome wide scan that discerns polymorphisms distributed throughout the chromosome. A QTL may be defined as any locus that influences the variability in a quantifiable phenotype (Rogers et al., 1999).

The variance component method is used to conduct linkage analysis. According to this technique population wide variance in a trait is due to genetic and non-genetic agents. Relationship between traits of family members can be addressed by dividing the agents that cause variance into i) a QTL associated with a chromosomal marker,

ii) QTLs not related to the segment of interest, and iii) shared and individual environmental factors (Comuzzie et al. 2001).

The concept of identity by descent (IBD) is the basis for linkage analysis. According to IBD two alleles at a locus are identical by descent if they are inherited from a single common ancestor without any new mutation (Rogers et al., 1999). Sharing of an IBD allele provides information about linkage. The expected IBD sharing between two related individuals is twice the kinship (Φ). Kinship is the probability of two genes from the same chromosomal loci selected from two individuals to be identical by descent. The variance elements can be computed by modeling covariance among members of the same ancestry:

$$\Omega = \Pi \sigma^2_q + 2 \Phi\sigma^2_a + I \sigma^2_e$$

Where Ω = covariance matrix for kindred

Π = IBD matrix for related members at a specific marker

σ^2_q = additive genetic variance owing to a QTL associated to a marker

Φ = kinship matrix

σ^2_a = residual (non-genetic) additive variance

I = identity matrix

σ^2_e = environmental difference related to an individual

The variance component model is implemented in the SOLAR program (Almasy and Blangero, 1999). The maximum likelihood method is used to verify the effect of a QTL. The null hypothesis, that additive genetic variance attributable to a QTL is zero is tested by comparing the likelihood of a model whose QTL is estimated with the likelihood of a model whose QTL is restricted to zero. This hypothesis testing generates a

logarithm of the odds (LOD). A LOD score of three or more is generally taken to be a strong predictor of the proximity between two loci. Twice the difference of the natural logarithmic likelihoods of these models is asymptotically distributed as a ½ : ½ mixture of chi-sqaure distribution with one degree of freedom (Self & Liang, 1987).

Bivariate genetic analysis was employed to examine the genetic correlations between two phenotypes. Genetic relationship is the degree of pleiotropy or the percentage of genes shared by two quantifiable characteristics. In other words, genetic correlation is an estimate of the proportion of shared genes common to both traits. A bivariate phenotype may be due to a person's measurable phenotype, population average, additive genetic effects and environmental consequences. A 2 × 2 covariance matrix provides the phenotypic covariance which is defined by the following equation:-

$\Omega ab = 2\Phi\rho G\sigma ga\sigma gb + I\rho E\sigma ea\sigma eb$,

where a and b assume the values of 1 or 2, and ρG and ρE are the additive genetic and environmental correlations between the traits, respectively. `

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